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THE

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PHOTOPERIODIC INDUCTION OF DIAPAUSE IN AN INSECT[1]

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Diapause is defined as a state of arrested development in which the arrest is enforced by a physiological mechanism rather than by concurrently unfavorable environmental conditions. Although not maintained directly by environmental factors, diapause is apparently induced, and in many species also terminated, in response to environmental stimuli. Most insect species are capable of diapause at some stage in the life cycle; embryonic, larval, pupal, and adult diapauses have been reported. Since the classical study of Kogure (1933) on the role of photoperiod in the induction of embryonic diapause in the silkworm, *Bombyx mori* L., daylength has been reported as a major inducing factor in the diapause of many other species. The recent profusion of reviews and symposia on the subjects of diapause and photoperiodism makes a detailed review of literature undesirable here; see Lees (1955, 1959, 1960), Bunning (1960), and Harker (1960b, 1961).

The present study was undertaken to demonstrate some of the characteristics of photoperiods effectively inducing diapause in the European corn borer, *Ostrinia nubilalis* (Hbn.). Larval diapause in this species has been shown to be a response to short days and low temperatures (Mutchmor and Beckel, 1959; Beck and Hanec, 1960). These earlier studies involved measurement of the effects of ecologically possible photoperiods, *i.e.*, a 24-hour total cycle. Under such limited experimental conditions, it was not possible to determine the relative importance of the light and dark phases of the over-all photoperiod, because one phase could not be varied except at the expense of the other. Neither was it possible to determine whether or not the photoperiodic response involved an endogenous circadian rhythm within the organism, as postulated by Bunning (1960) or a non-circadian "interval timer," as postulated by Lees (1960a, 1960b). The experimental work reported below was designed to contribute to the clarification of these problems.

Materials and Methods

The European corn borers used in this study were from a restricted natural population occurring near Madison, Wisconsin. The use of a defined population

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was necessary because of the demonstration of significant differences in photoperiodic responses among different geographical populations of this species (Beck and Apple, 1961). Overwintering borers were collected from the field in the fall of the year, and were stored at 4° C. As needed, groups of the stored borers were placed at 30° C. for pupation and emergence. The progeny of these insects were used in the experiments described below.

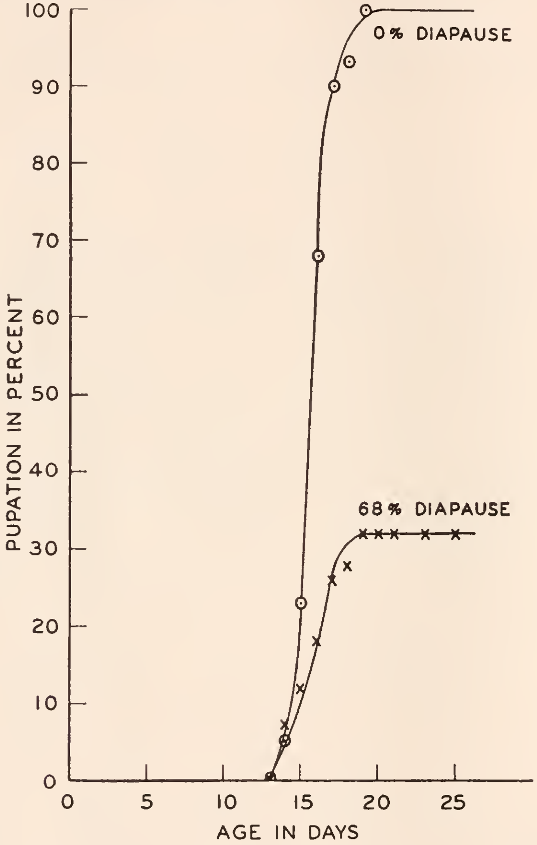

FIGURE 1. Typical pupation records of experimental populations of the European corn borer, illustrating the method of determining incidence of diapause.

The experimental borers were reared aseptically on purified diets, according to the rearing techniques described by Beck and Smissman (1960). Photoperiod experiments were run at 30° C., and the larvae were exposed to the experimental photoperiods continuously from an age of less than 24 hours. Each experiment involved the use of 250 newly hatched larvae. The larvae were divided into 5 groups of 50 each; one group was reared in continuous dark as the experimental control; the other 4 groups were exposed to the experimental conditions and were treated statistically as replicates. Because of mortality and microbial contamination, the final number of insects in each replicate and the control varied from 40 to 46. As the larvae reached maturity, daily pupation records were taken. Diapause incidence was measured as the per cent of mature larvae that failed to pupate. This was determined on the basis of the sigmoid configuration of the pupation record (Fig. 1); when the curve had remained unchanged for several days, pupation was considered to have been completed. The remaining mature larvae were considered to be in diapause.

The experiments were carried out in B.O.D. constant temperature incubators that had been modified to incorporate a thermistor temperature control system (Thermistemp Temperature Controller Model 71, Yellow Springs Instrument Company, Yellow Springs, Ohio). Control of photoperiod was effected through the use of 7-day cycle programmers wired to two 14-watt fluorescent lights installed in the incubator. In experiments involving temperature changes, a 24-hour temperature cycle was effected by using a clock motor to drive the thermistor temperature control unit through a prescribed cycle. The performance of the cycling apparatus was verified by the use of a recording thermograph.

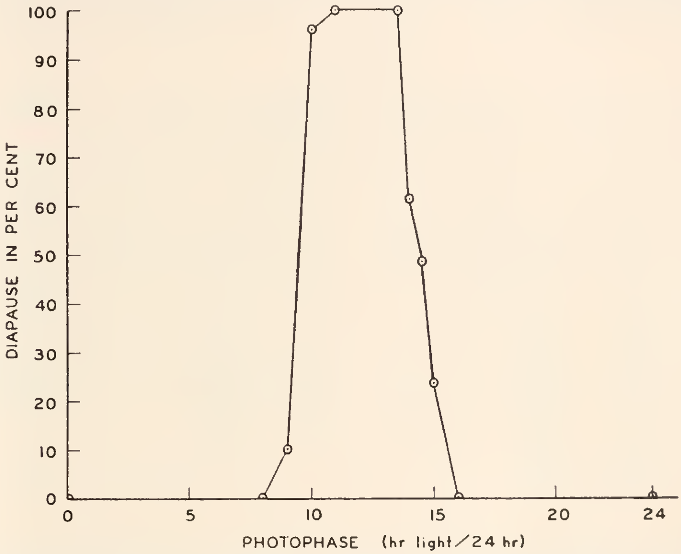

FIGURE 2. Effect of photophase duration on diapause incidence among European corn borer larvae reared under 24-hour photoperiods.

In the discussion of the results, which follows, the term *photoperiod* is restricted to refer to the total cycle composed of a period of light and a period of dark. The period of light within the photoperiod is referred to as the *photophase;* conversely, the dark portion of the photoperiod is termed the *scotophase*. This terminology is recommended as a means of avoiding the usual ambiguous use of the term photoperiod. It has been commonly used to refer to (1) the total light/dark cycle, and (2) only the light portion of the total cycle. Since the structure of the word implies a periodicity involving light, and since the only periodic alternative to light is dark, the term should be used only in the sense of the total light/dark cycle.

RESULTS

At an incubation temperature of 30° C. and a photoperiod of 24 hours, the effect of photophase duration on the incidence of diapause in the European corn borer is shown in Figure 2. The effective range of photophases was between 8 and 16 hours, with diapause incidence exceeding 90% only under photophases of from 10 to 14 hours. The borer is a "long-day" insect, in the classical sense of the term (Dickson, 1949; Lees, 1952; Otuka and Santa, 1955). It should be noted, however, that the term "long-day insect," in the sense of an insect that develops without interruption under the influence of 24-hour photoperiods containing a relatively long photophase (> 15 hr.), is a misnomer. Abnormally short photophases (< 9 hr.) also result in uninterrupted development in this and some other species. The diapause incidences obtained (Fig. 2) were in agreement with those reported in earlier studies of diapause in the European corn borer (Mutchmor and Beckel, 1959; Beck and Hanec, 1960), and are of interest to the present study only in that they delimit the responses of the insect to naturally occurring photoperiods.

TABLE I

Effect of photophase: scotophase ratios on incidence of diapause among European corn borer larvae

Photophase:Scotophase = 1:1				Photophase:Scotophase = 2:1				Photophase:Scotophase = 1:2			
Photo. (hr.)	Scoto. (hr.)	Total (hr.)	Diapause (%)	Photo. (hr.)	Scoto. (hr.)	Total (hr.)	Diapause (%)	Photo. (hr.)	Scoto. (hr.)	Total (hr.)	Diapause (%)
6	6	12	<5	10	5	15	<5	4	8	12	<5
8	8	16	<5	12	6	18	<5	5	10	15	32
9	9	18	78	14	7	21	<5	6	12	18	95
10	10	20	92	16	8	24	<5	7	14	21	65
12	12	24	100	20	10	30	31	8	16	24	<5
14	14	28	85	24	12	36	65				
16	16	32	50	28	14	42	<5				
18	18	36	19								
20	20	40	<5								

Diapause incidence under 24-hour photoperiods approached a maximum when the photophase: scotophase ratio was approximately 1:1, and approached a minimum at ratios of either 1:2 or 2:1. Diapause incidence under photoperiods displaying different photophase: scotophase ratios is shown in Table I. One-to-one ratios were ineffective when the phase durations were either under 8 or over 16 hours. Diapause incidence was very high when both photophase and scotophase were from 9 to 14 hours, which is similar to the responses obtained in the series of 24-hour photoperiods (Fig. 2). When the duration of the photophase was twice that of the scotophase (2:1 series, Table I), diapause was induced only when the scotophase was from 10 to 12 hours. When the scotophase was twice as long as the photophase (1:2 series, Table I), diapause was evoked only by scotophases of from 10 to 14 hours. Total photoperiod duration did not appear to influence the incidence of diapause in these experiments; nor did the phase ratios appear to be of any significance. Diapause induction was closely associated with scotophases of from 10 to 14 hours, with maximum effectiveness at 12 hours. These findings are in agreement with those of Danilyevsky and Glinyanaya (1950), who worked with *Acronycta* spp. Otuka and Santa (1955), experimenting with *Barathra brassicae* L., concluded that, although the absolute length of the phases was of importance, phase ratio also influenced diapause induction; their data on this point are not all convincing, however, as they tested only 1 : 1 ratios.

A series of experiments was run in which photophases of 10, 12, and 14 hours were combined with a wide range of scotophases (Fig. 3). At these photophases, the range of diapause-inducing scotophases was relatively narrow; scotophases of from 10 to 14 hours were required to produce a diapause incidence of 90 or more per cent. The three response curves shown in Figure 3 did not prove to be different to a statistically significant degree. Maximum diapause incidence was induced by a 12-hour scotophase in each case.

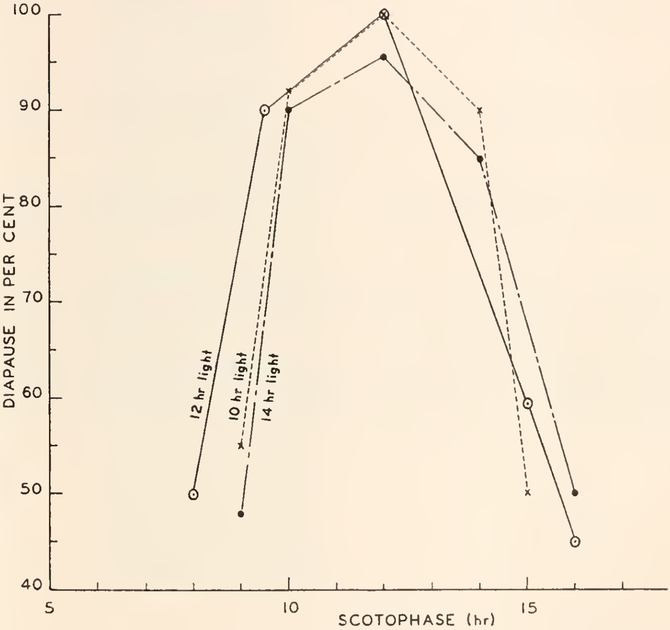

FIGURE 3. The effect of scotophase duration on the incidence of diapause in European corn borer larvae reared under photophases of 10, 12, or 14 hours.

Diapause incidence among borers reared under scotophases of 10, 12, and 14 hours, combined with a wide range of photophases, was also measured (Fig. 4). A 12-hour scotophase was significantly more effective than 10- or 14-hour scotophases. The 12-hour scotophase induced a high incidence of diapause when it was combined with any photophase of from 5 to 18 hours duration. A photophase of more than 28 hours was required to reduce the diapause incidence to below 50%, when the experimental photoperiod included a scotophase of 12 hours.

Phase durations required to induce a diapause incidence of 90 or more per cent are shown in Figure 5. The 90% response curve describes an ellipse, within which

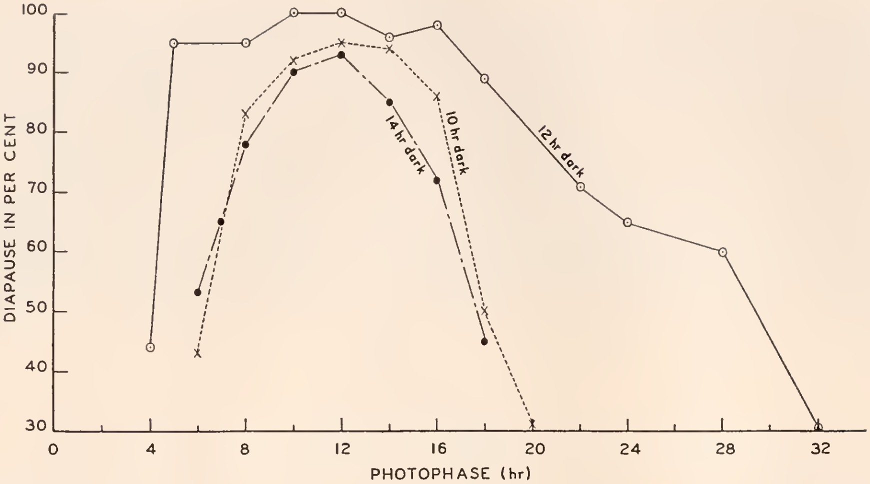

Figure 4. Effect of photophase duration on the incidence of diapause among European corn borer larvae reared under scotophases of 10, 12, or 14 hours.

diapause incidence was greater than 90%. The long axis of the ellipse lies along the 12-hour scotophase coordinate, and the widest part of the response zone falls between the 11- to 13-hour photophase and 10- to 14-hour scotophase coordinates. These results, combined with those shown in Figures 3 and 4, suggest that diapause is most efficiently induced by a 24-hour photoperiod containing a 12-hour scotophase.

The characteristics of photoperiods inducing a high incidence of diapause in the

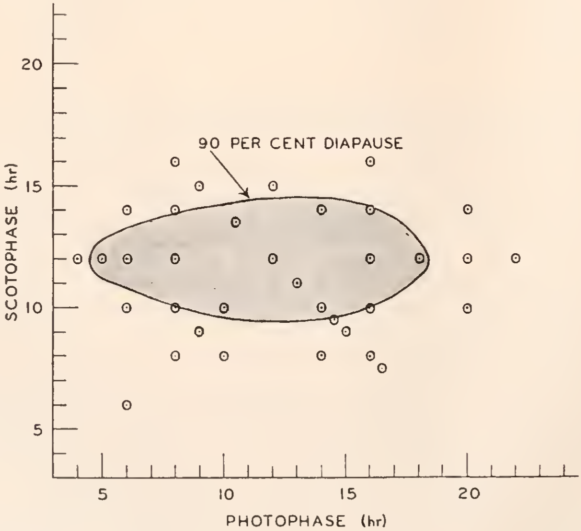

Figure 5. Photoperiodic requirements for the induction of a 90 or more per cent incidence of diapause among European corn borer larvae. (Plotted points represent photoperiods tested experimentally.)

European corn borer cover a much wider range of phases than has been found in some other lepidopterous species (Dickson, 1949; Bull and Adkisson, 1960). The response range of the mite, *Metatetranychus ulmi,* is fundamentally different from those of the lepidopterous species tested, in that long scotophases (12 to 24 hours) tended to promote diapause incidence, and long photophases tended to suppress diapause. A 12-hour scotophase was found to be as effective as a 24-hour scotophase, indicating a very broad range of maximum effectiveness (Lees, 1953, 1955).

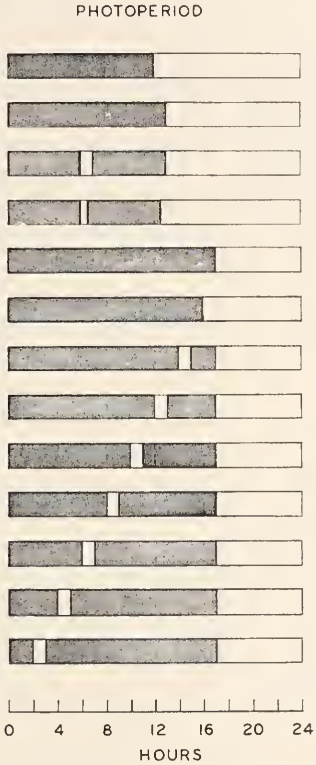

FIGURE 6. Effect of one-hour light interruptions of the scotophase on diapause induction in the European corn borer (24-hour photoperiod).

Photoperiodic induction of diapause in the European corn borer is dependent upon the actual duration of the photoperiodic phases, with the duration of the scotophase being far more critical than that of the photophase. This situation appears to prevail in other photoperiodically responsive arthropods as well. Lees (1953) found that a scotophase of 12 hours would induce the production of diapause-eggs in *Metatetranychus ulmi* even when accompanied by photophases up to 36 hours long. Tanaka (1950), working with the Chinese Tussar-silkworm *Antheraea pernyi,* reported that a 13-hour scotophase was effective in inducing diapause under photoperiods containing photophases as long as 59 hours. All of these results indicate that, although minor species differences exist, diapause in "long-day" insects is a response to scotophases of about 12 hours duration, within a surprisingly broad range of photophases.

A series of experiments was run to test the effect of interrupted scotophases on the incidence of diapause (Fig. 6). In the first group of such experiments, an hour of light was inserted in the middle of the 13-hour scotophase of a 24-hour photoperiod. Diapause incidence was negligible under such a photoperiodic regime, whereas an uninterrupted scotophase of 12 or 13 hours induced a diapause incidence of well over 90%. Interruption of the scotophase by as little as 0.5 hour of light greatly reduced diapause incidence. The sensitivity of the borer to an interruption of the scotophase is greater than that reported for a number of other insect species, in which interruptions of from two to three hours were required to reduce the diapause-inducing effect of a 12-hour scotophase (Dickson, 1949; Lees, 1953; Danilyevsky and Glinyanaya, 1950; Bunning, 1960).

Scotophases of 17 hours in a 24-hour photoperiod were systematically interrupted by a one-hour light period (Fig. 6). Uninterrupted scotophases of 16 and 17 hours did not induce a significant level of diapause. When the one-hour light break came at the end of 10, 12, or 14 hours of dark, a significant incidence of diapause was induced. Once again, the highest incidence was associated with 12 hours of uninterrupted dark. When the interrupting light occurred after either 6 or 8 hours of dark, no diapause was observed. After either two or four hours of dark, an hour of light followed by 14 or 12 hours of dark resulted in a diapause incidence of about 50%. Apparently, a 12-hour dark period is less effective if preceded by a four-hour dark period than if followed by a four-hour dark period.

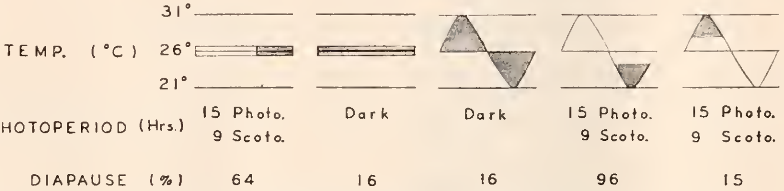

FIGURE 7. Effect of combined photoperiod and thermoperiod on the incidence of diapause in the European corn borer.

Using the cabbage worm, *Pieris brassicae,* Bunning and Joerrens (1960) also determined the effects of interrupted scotophases on diapause incidence. Although the experimental insect was also a "long-day" form, systematic interruption of the scotophase with one-hour light periods produced a different pattern of response. They found that diapause was prevented by light interruptions occurring about 15 hours after the beginning of the photophase, regardless of the total length of the scotophase or the position of the interruption within the scotophase. The results with the European corn borer (Fig. 6) show no such relationship, and diapause incidence is obviously dependent upon an uninterrupted dark period of about 12 hours, with the response being only slightly influenced by the position of the 12-hour dark period within the total photoperiod.

The previous finding that diapause incidence under a given photoperiod is temperature-sensitive (Beck and Hanec, 1960), and the finding that diapause incidence is closely dependent upon the duration of the scotophase, led to the hypothesis that the temperature sensitivity of the diapause reaction is associated with the temperature during the scotophase. This was tested by the experimental series shown in Figure 7. Low temperatures during the 9-hour scotophase caused a very high incidence of diapause, whereas low temperatures during the photophase produced no more diapause than did the control conditions of continuous dark. The results clearly imply that diapause induction in the European corn borer involves a scotophasic temperature sensitivity.

Because of the reported effects of light and photoperiodicity on growth rates and a variety of morphogenic processes (Muller, 1957, 1958, 1960; Ball, 1958) growth records were maintained in all experiments conducted in this study. In an earlier paper (Beck and Hanec, 1960) it was reported that the series of 24-hour photoperiods tested had no measurable effects on the rate of larval growth of the European corn borer. In the present study, a much wider range of photoperiods was studied, but no growth rate effects were detected. At the rearing temperature employed (30° C.,) the larvae attained the fifth instar at an age of about 10 days, prepupal stage at 11 to 13 days; pupation occurred from the thirteenth day, and pupation of the non-diapause portion of the experimental populations was 50% completed by about the seventeenth or eighteenth day. This developmental schedule did not appear to be materially altered by any photoperiod tested, except, of course, that the larvae entering diapause did not pupate.

Discussion

From the results of this study, some of the characteristics of the photoperiodic reactions of the European corn borer may be deduced. The induction of diapause is obviously associated with the periodic occurrence of 12-hour scotophases during the 11-day developmental period of the larva (at 30° C.). Within rather broad limits, the actual duration of the scotophase was found to determine the incidence of diapause, regardless of the relative proportion of the photoperiod occupied by the scotophase.

Following exposure to a 12-hour scotophase, the borer is apparently refractory to a second dark "stimulus" for a period of from 4 to 5 hours. This interpretation is based on the results of two types of experiments. First, the shortest diapause-inducing photophase was found to be between 4 and 5 hours in duration (Fig. 4). This might be interpreted as meaning that the "light reaction" requires from 4 to 5 hours for effective activation, were it not that the results of the interrupted scotophase experiments are inconsistent with such a conclusion (Fig. 6). These experiments showed that light durations of an hour or less are effective. They also indicate the existence of a dark-refractory period following a 12-hour scotophase; the photoperiodic regime of 12 hours dark, 1 hour light, 4 hours dark, and 7 hours light resulted in a diapause incidence of 88%. Statistical analyses showed that such a response was not significantly different from the response to a simple photoperiod composed of a 12-hour scotophase and 8-hour photophase. It would seem that the borers failed to respond to the added four hours of dark.

The effect of a photophase of several hours is apparently no more than the effect of a photophase of but one hour. Diapause incidences of about 50% were obtained when a 17-hour scotophase was divided into a short period (two- or four-hour), an hour of light, and a long dark period (14- or 12-hour) (Fig. 6). Since a significant incidence of diapause was observed, the short dark period was not followed by a dark-refractory period. The long dark periods had a diapause-inducing effect, as expected, but were followed by a second effective period of light. The over-all effect of such a photoperiodic regime was that there were two effective periods of light (1 and 7 hours) for every effective scotophase, thus reducing the diapause-inducing effect to the photoperiod.

Even with 12-hour scotophases, diapause incidence declined slowly as the length of the total photoperiod exceeded 30 hours (Fig. 4). This effect is probably determined by the number of photoperiods occurring within the 11-day larval developmental period. A 12-hour scotophase combined with a 5-hour photophase produces a 17-hour photoperiod, and 15.5 such photoperiods can occur in 11 days (264 hours). However, when a 12-hour scotophase is combined with a 32-hour photophase, the photoperiod is 44 hours, and only 6.0 such photoperiods can occur in 11 days. The gradual decline of diapause incidence observed with photoperiods of increasing length is interpreted as being explicable on the basis that the incidence of diapause tends to be proportional to the number of 12-hour scotophases experienced during larval development.

Assuming that the induction of diapause is dependent upon the occurrence of a required number of effective scotophases and photophases, it should be possible to devise a system of predicting the incidence of diapause under any given photoperiodic regime. Such a predictive scheme would be based on the accumulation of diapause-effective hours of light and dark over the period of larval development, in a manner comparable to the temperature accumulations that have widespread use in phenological predictions. A number of systems of photoperiodic accumulation were devised. Highly significant coefficients of correlation between per cent diapause and photoperiodic accumulations were obtained by nearly all of the methods tested. However, the schemes were devised to fit the data at hand, and in spite of such bias, serious discrepancies were apparent between "predicted" and observed results in a few crucial experiments. For these reasons, the accumulation methods tried were concluded to be without real meaning. Until more is known about the dynamics of both the "light" and the "dark" reactions, an empirical system of predicting diapause incidence is not likely to be of much fundamental significance.

The over-riding importance of a scotophase of about 12 hours and an effective light flash of only one hour in the induction of diapause may be interpreted to support the conclusion of Lees (1960) that the physiological mechanisms involved behave as an interval timer. The diapause-inducing photoperiods tested in this study were not confined to a periodicity of 24 hours, or any multiple thereof, or to any other specific cycle duration. These findings lend little support to the hypothesis that diapause is in response to the effects of photoperiods on an endogenous circadian rhythm. On the other hand, Figures 3, 4, and 5, above, may be interpreted as lending at least feeble support to the hypothesis that photoperiodic induction of diapause involves a circadian function, in that the most effective range of photoperiods centered around a 12-hour photophase as well as a 12-hour scotophase. The tendency for diapause induction to be associated with a 24-hour periodicity was much less well defined than was its dependence on a 10- to 14-hour scotophase.

Diapause, itself, cannot be a rhythmic function: it occurs but once per individual. Whether or not one or more of the contributing physiological events involves a circadian rhythm has not been demonstrated. Feeding behavior activity cycles have been implicated in the induction of diapause in the beetle, *Leptinotarsa decemlineata* (DeWilde *et al.*, 1959), but the photoperiodic effects could not be fully explained solely on the basis of the effects of photoperiod on feeding. Modification of photoperiodic effects by experimental alteration of feeding behavior was also reported by Muller (1957), who worked with a number of species of the homopterous genus, *Euscelis*. Circadian rhythmicity and an influence of photoperiod on such activities as locomotion and eclosion have also been reported (Harker, 1960a; Pittendrigh and Bruce, 1959). A relationship between circadian cycles, neurosecretory cycles, and diapause induction has thus far eluded demonstration, however. Whether diapause is the result of photoperiodic induction of elaboration of a "diapause hormone" (Hasegawa, 1957; Lees, 1959b; DeWilde and Boer, 1961), or a photoperiodically induced biochemical failure in the morphogenic chain of events remains to be determined.

SUMMARY

1. The European corn borer, *Ostrinia nubilalis*, is a so-called long-day insect, larval diapause being induced by naturally occurring photoperiods containing scotophases of from 10 to 14 hours.

2. Diapause induction was found to be dependent upon the actual number of hours of the photoperiodic phases. The duration of the scotophase was far more critical than that of the photophase. A 12-hour scotophase was of maximum effectiveness when combined with photophases of from 5 to 18 hours. Significant incidence of diapause occurred when a 12-hour scotophase was combined with photophases of from 4.5 to 32 hours.

3. Diapause induction is a temperature-sensitive phenomenon, with the incidence of diapause tending to be inversely proportional to the ambient temperatures occurring during the scotophase.

4. Interruption of the scotophase by a one-hour period of light modified the photoperiodic response, the effect depending on the position of the light interruption within the scotophase. The effects were interpreted as a demonstration that the insect's photophasic requirement is satisfied by a one-hour light period, but that longer photophases are normally required because of a dark-refractory period following 12-hour scotophases.

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AGGREGATION TERRITORIES IN THE CELLULAR SLIME MOLDS[1]

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The size of the area or territory which encompasses the amoebae that enter into an aggregate of a cellular slime mold is related to the process of initiation of aggregation as well as the size of the sorocarp. Each territory represents one aggregation and therefore one initiation event, and the size of any one fruiting body, or sorocarp, is primarily determined by the number of amoebae that enter an aggregation center. It is true that some of the cells may divide after the beginning of aggregation (Wilson, 1952; Bonner, 1960) but since all intake of food ceases some time before aggregation, there is no increase in bulk during the morphogenetic phases of the life-cycle, that is, while the aggregated cell mass progressively becomes transformed into a fruiting body.

It will be shown that regardless of the density of the amoebae, the territory size, under controlled environmental conditions, remains constant for any one species. This means that sorocarp size is largely controlled by cell density and that the problem of initiation is identical with the problem of the establishment of these rigid aggregation territories.

MATERIALS AND METHODS

A simple method was used for the control of the amoeba density within a culture by controlling, in turn, the amount of bacterial food supply. Two per cent Bacto agar containing 6.2 μg./ml. of dihydrostreptomycin sulphate (Lilly) was poured into plastic Petri dishes which were marked on the bottom surface with squares of .102 cm^2. A few loopfuls of *Escherichia coli* taken from a stock culture (grown on 1% dextrose, 1% peptone, 2% Bacto agar) were placed in 2 to 7 ml. of sterile distilled water. The density of the suspension was then determined in a Bausch & Lomb "Spectrometer 20" at 550 mμ. Appropriate dilutions were made to achieve a particular density and .09 ml. of this final suspension was evenly spread (with the help of an electric turntable and a sterile, bent glass rod) on the surface of one of the streptomycin agar Petri plates. In this way it was possible to obtain a range in food supply on the plates from approximately 500,000 bacterial cells/mm.^{2} (= an optical density of 6.0) to 5000 bacterial cells/mm.^{2} (= an optical density of 0.1). The spores of the slime mold were inoculated in three marked spots on each plate with a very fine, glass needle with a tip rounded in a small glass bead about .5 mm. in diameter, and consequently the inoculation points were confined to small, limited areas. Except for special

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experiments involving controlled temperatures and light, the dishes were incubated on a table in the center of the laboratory (25 ± 3° C.). This was done after determining that the results were identical with those done at constant 24° C. conditions in continuous light, and in 12 hours of light alternating with 12 hours of darkness. Also in order to check the possibility that the streptomycin might be affecting the results in some way, some controls were run without the streptomycin, and no difference could be observed in the morphology or the size of the aggregation territories.

The counts of fruiting body density were made by inverting the Petri dish under a dissecting microscope and counting the number of fruiting bodies in 5 separate .102-cm.^{2} areas and averaging the results. The mean aggregation territory was calculated directly from the sorocarp density. The radius of the aggregation territory was determined by considering each territory to be a circle. The size of fruiting bodies was determined two ways: in the case of very small ones the number of spore and stalk cells was counted directly, and in larger ones a camera lucida drawing was made of the stalk so that stalk lengths could be accurately determined.

RESULTS

Sorocarp size

According to the original description of the various cellular slime molds, each has its characteristic size. These sizes are usually indicated as stalk lengths, and under normal culture conditions this is an appropriate measure, although it is possible, by increasing the humidity of the air and decreasing the solute concentration of the agar, to obtain stalks of great length (Bonner and Shaw, 1957). For instance, in *Dictyostelium mucoroides* the largest are described as having stalks of 10 to 50 mm. or more (Raper, 1951) while under the specially humid conditions we obtained stalks up to 220 mm. in length. But this involved no increase in cell number; it is merely that the humid conditions favor prolonged migration and the transfer of the majority of amoebae into stalk cells, leaving very few spores. We are not concerned here with this aspect of size, but rather with size changes which reflect changes in cell number (or in dry weight).

As Raper (1951) has stressed, *D. mucoroides* may best be described as a complex, rather than a species, for the variability among strains is great, size being one of the significant variables. At one extreme there are the large forms typified by *D. giganteum* of Singh (1947) and at the other there is *D. minutum* of Raper (1941). The range in stalk height (under conditions which reflect cell number) is from .5 to 50 mm.

In the case of *D. discoideum* all the naturally occurring strains described thus far are all roughly the same size (a stalk height of 1 to 3 mm.) although Sussman (1955) has produced a mutant ("fruity") by U.V. radiation that is considerably smaller.

The genus *Polysphondylium* contains two species which differ significantly in size. The larger *P. violaceum* has a stalk length of about 5 to 30 mm. (Olive, 1902) while *P. pallidum* is roughly half the size.

D. lacteum, which has round rather than elliptical spores, is also a small species (.5 to 1.5 mm.). *Acytostelium leptosomum* (Raper and Quinlan, 1958), another round-spored form, is small (1 to 1.8 mm.) but presents rather a special case in that the stalk is non-cellular and consists of a simple delicate cylinder of cellulose. The ultimate in small-size in the cellular slime molds is *Protostelium* (Olive and Stoianovitch, 1960) which consists of one cell which builds its own stalk (.07 mm. in height).

In all these cases the stalk length reflects size under optimum culture conditions. In the case of the larger forms this is a rich medium which produces a profusion of bacterial food supply. It is a well known and every-day observation that if a medium is used which supports poor bacterial growth, these large species will produce smaller sorocarps. Arndt (1937), in fact, made a series of observations of cultures with different densities of bacterial food supply, and came to the conclusion that the size of the fruiting body was directly dependent upon amoeba density. As will be seen presently, this study is partly a quantitative exploitation of this commonplace observation.

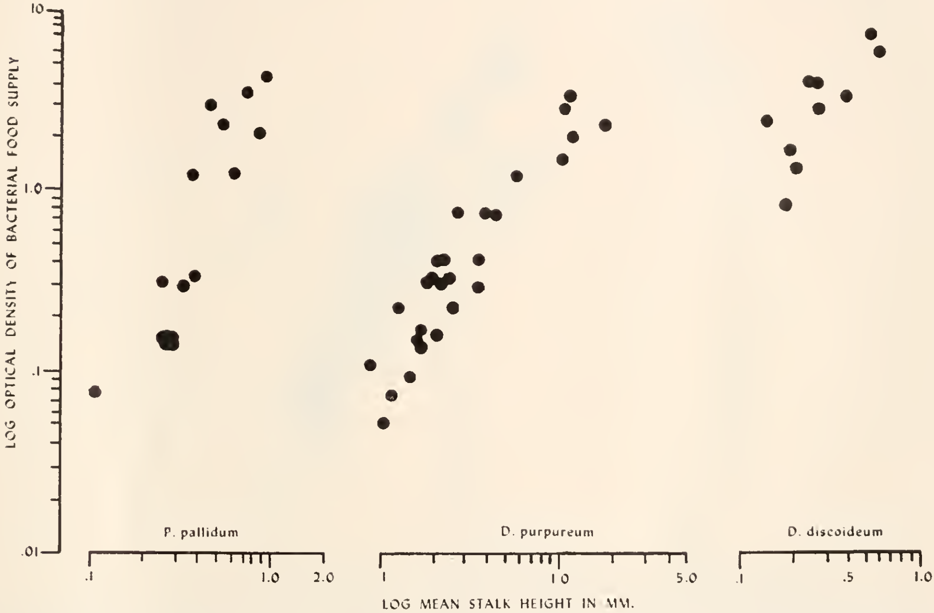

FIGURE 1. A graph showing the density of the bacterial food supply plotted against the mean stalk height which is used as an index of sorocarp size. Each point is an average of 20 stalks.

Another frequent observation, repeatedly emphasized by Raper (*e.g.* 1951), is that the smaller species will not grow in rich media, but require dilute media. This is true for all the small forms mentioned above (*D. minutum, P. pallidum, D. lacteum, A. leptosomum*). In other words, for these species, the maximum size seems to be determined by the density of the bacterial food supply; there is an upper limit in the amount of food, above which all development is inhibited, possibly because of the production of inhibitory substances by the abundant bacteria.

With this in mind, different species were grown on different known concentrations of *E. coli* on streptomycin agar. As can be seen from Figure 1, the size of the sorocarps increases with the increase in bacteria. The range indicated here only shows the lower limit of the species tested, but fails to show the upper limit.

Note that for the three species shown in Figure 1, each has a different minimum threshold. Some studies were also made of *D. minutum* which, as just mentioned, has a low maximum threshold (*i.e.,* does not develop with a rich food supply, probably because excessive bacterial growth produces inhibitory substances). It was a surprise to discover that it had a high minimum threshold necessary for aggregation. In other words it is a form which can only develop in a very restricted range of food densities. Other forms, such as *D. purpureum,* are much less particular and can adapt to a wide range of concentrations, and can produce, as a result, a wide range of size in their fruiting bodies from ones much smaller than *D. minutum* to ones very much larger. This leads to an important point as far as *D. minutum* is concerned: one of the reasons for its small size is the fact that it normally develops in a restricted range of food density and because of the low value of this particular range, the mean sorocarp size is correspondingly small. There are undoubtedly other factors which also may limit size in *D. minutum,* a matter which is under further investigation.

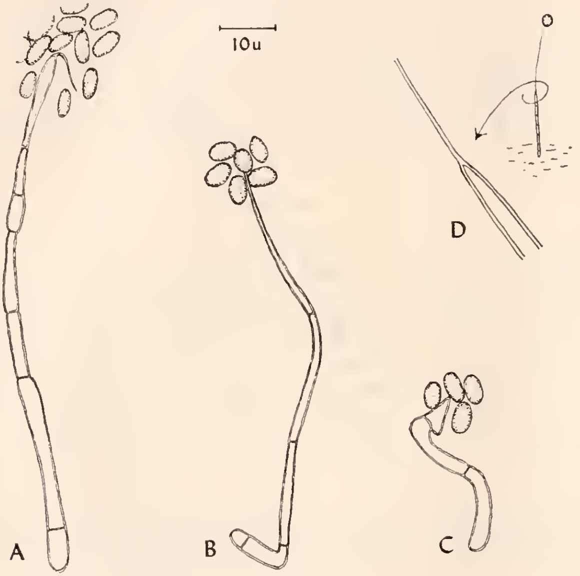

FIGURE 2. Camera lucida drawings of small sorocarps. A. *D. purpureum* with 7 stalk cells and 45 spores (not shown). Note the wisp at the end of the stalk. B. *P. pallidum* with 5 stalk cells and 6 spores. C. The smallest sorocarp obtained. It is *P. pallidum* with 3 stalk cells and 4 spores. D. *D. lacteum* showing a small stalk which midway becomes acellular.

In general, on any one culture dish, at any one bacterial density, the sorocarps were remarkably uniform in size. At the minimum thresholds for each species it was of interest to examine the morphology of the smallest sorocarps for any possible effects caused by the size reduction. In some species the stalk cells appeared somewhat coarse and club-like (*D. mucoroides, D. purpureum, D. discoideum*) while in others the cells were beautifully tapered (*P. pallidum, P. violaceum, D. lacteum*) (Fig. 2). In the case of *D. lacteum* the smaller sorocarps had an acellular tip, giving them, at least at their anterior end, an appearance almost exactly resembling *Acytostelium*. This is not true of the larger sorocarps of *D. lacteum*. Occasionally in *D. purpureum* it was possible to observe sharp acellular wisps of stalk material at the anterior end of the stalk. The smallest fruiting body encountered in these studies of all the species examined was a seven-cell sorocarp of *D. purpureum* (three stalk cells, four spores).

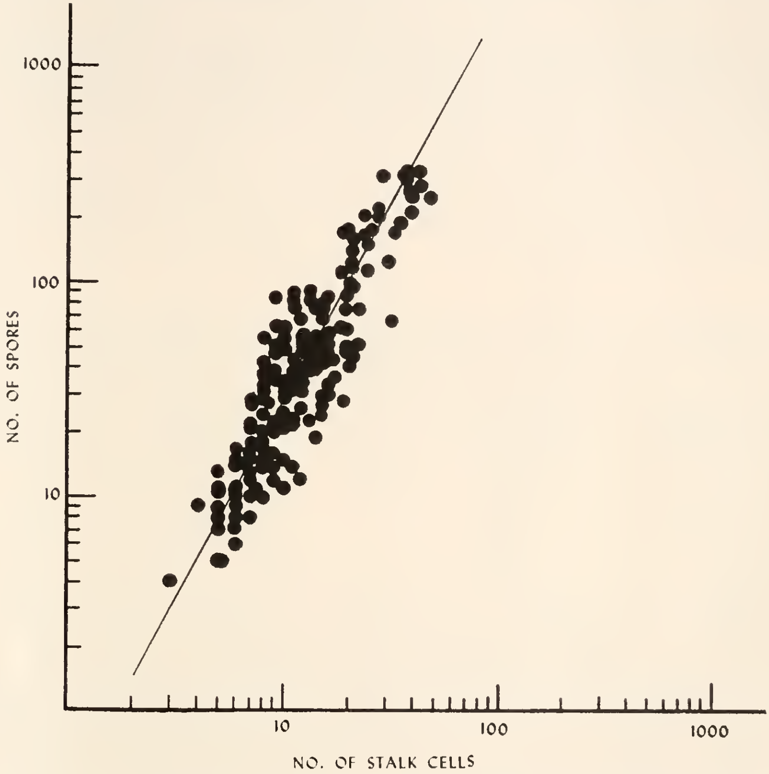

FIGURE 3. The log of the number of spores is plotted against the log of the number of stalk cells. These points are a composite of *D. purpureum, D. mucoroides,* and *P. pallidum*, as they showed no discernible difference among them.

Since it was possible to count the cells in small fruiting bodies, a study was made of the proportions of stalk to spore cells, this method having many advantages over the less direct ones previously devised (Bonner and Slifkin, 1949; Bonner, 1957). A number of counts were made for *D. mucoroides, D. purpureum* and *P. pallidum* (these latter ones were unbranched because of their small size, but as they showed no significant difference among them, they have all been plotted together (Fig. 3). The character of the allometric relation is identical to the one previously described for larger pseudoplasmodia (Bonner, 1957).

Aggregation territories

While size and food supply (which is a reflection of amoeba density) are directly proportionate, fruiting body density is independent of size. That is, regardless of the density of amoebae, the size of an aggregation territory remains constant (Fig. 4). It is of interest to note that Arndt (1937) came to a similar conclusion, although he made no quantitative determinations to support his observations. To emphasize the point, it is space, not the number of cells, that is important in determining the size of an aggregation territory. If there are 100 cells in a unit space, they will produce one fruiting body of 100 cells; if there are 1000 cells then they will produce one fruiting body of 1000 cells. Of course this statement is oversimplified as it ignores the possibility of cell division and the fact that not all the cells may enter the aggregate but some are left behind (and in fact their number can be accurately determined).

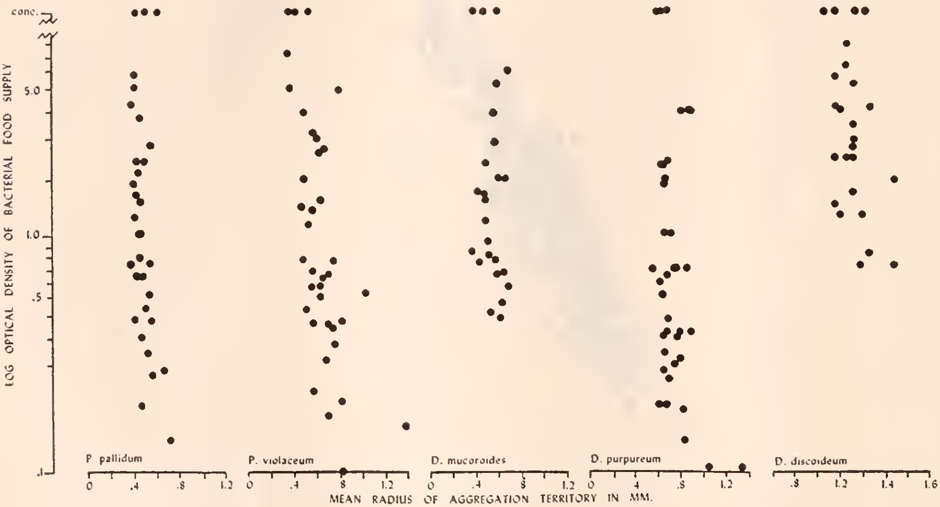

FIGURE 4. Five graphs for different species, showing the radius of the aggregation territory under conditions of different bacterial food densities. The top points labeled *conc.* are on 2% agar without streptomycin and a heavy layer of bacterial paste smeared evenly over the surface. Each point is an average of 5 squares (.102 cm.^{2}) on one culture dish.

Some experiments were also run at higher concentrations of bacteria to determine if the constancy of the size of the aggregation territory was affected by very high amoeba densities. This was first done on non-nutrient plates covered with a heavy layer of bacterial paste for all five species, and as can be seen in Figure 4 results compare with the more dilute plates. A further experiment was run on *D. purpureum* using full nutrient agar (10 gm. peptone, 10 gm. dextrose, .96 gm. $Na_2HPO_4 \cdot 12H_2O$, 1.45 gm. KH_2PO_4, 1000 ml. H_2O, 20 gm. agar) and the same diluted in half and by one fourth (with the exception of the agar content which remained at 2%). The results showed no obvious difference between the three concentrations of nutrients, and the range of twelve runs extended from a territory radius of .41 to .56 mm. which overlaps, but is significantly lower than the range using non-nutrient agar. This discrepancy is unexplained although there are a number of possibilities, such as the specific effects of the nutrients themselves on territory size, a possibility which will come up again in discussing the environmental factors which affect the aggregation territory.

As a check to determine if the same result could be obtained by using radically different techniques, two further experiments should be mentioned. In one the amoebae of *D. discoideum* were centrifuged free of bacteria and suspended in a salt solution (see Bonner, 1947) in van Tieghem cells with one end sealed with a coverslip. A microscope slide was then sealed to the other end of the cell, and the whole preparation inverted after the amoebae had settled on the coverslip. They were thus attached to glass and retained in small moist chambers which were incubated at 20° C. in the dark (except for hourly examinations under the microscope). At very high and very low amoeba concentrations the fruiting bodies were few and small, but in a large range of intermediate concentrations the territory size remained constant, while the mean stalk length tended to increase, as would be expected from previous results (Table I).

TABLE I

An experiment on territory size using D. discoideum *on coverslips in small moist chambers (van Tieghem cells)*

Amoeba density: amoebae/mm^2.	Mean sorocarp height in mm.	Total number of sorocarps per coverslip	Mean radius of the aggregation territory in mm.
246	.76	26	1.32
318	.77	23	1.41
618	1.08	22	1.44
1040	1.05	28	1.28
2570	.86	23	1.41

In the other experiment the liquid culture technique of Gerisch (1960) was employed and after the growth phase the amoebae of *D. purpureum* were harvested, plated out in different concentrations on the streptomycin agar plates and incubated in room conditions. This method has the advantage of making it possible to determine amoeba densities directly. If Figure 5 and Figure 4 are examined, it is obvious that the ranges of the territory size using the two techniques are comparable.

In order to examine the formation of aggregation territories in more detail, time lapse motion pictures were taken of the aggregation of *D. purpureum* at four different bacterial food densities on streptomycin agar. It was possible, in each of the cases, to determine the amoeba densities which were 54, 147, 174, and 356 amoebae/mm.^{2}, respectively (included in Figure 5). The only significant difference that could be observed among the four cases was that the resulting fruiting bodies were progressively larger as the amoeba density increases. As far as the time of formation of the aggregates and the general blocking out of the territories, they were all similar. For instance in none of the cases was there any

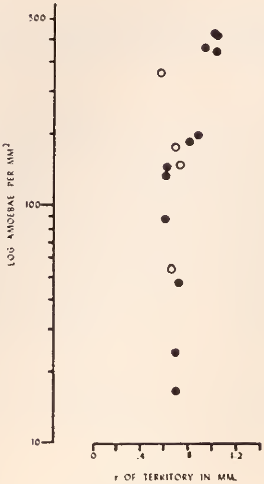

FIGURE 5. A graph showing the radius of the aggregation territory at different known amoeba densities for *D. purpureum*. The solid dots involve the Gerisch (1960) liquid culture technique and the hollow circles were taken from a motion picture of cultures prepared by the streptomycin agar technique described in Materials and Methods.

evidence of the formation of more numerous territories, some of which would then disappear. Each center, once formed, remained stable and there was no subsequent disintegration.

If the rate of appearance of new centers is plotted against time for each of the amoeba densities, it is clear that they do not appear steadily, but with considerable irregularity. However, under these conditions of constant illumination there was no evidence of bursts of aggregation such as Shaffer (1958) obtained with alternate darkness and light.

As is evident from Figure 4, each species has a characteristic territory size. They are listed in order of magnitude for the various species in Table II. A few determinations were also made of *D. minutum* and *D. lacteum*, which indicated that they are both on the lower end of the scale. This means that to a minor extent the small size of these two species and *P. pallidum* might be accounted for by their small territory size.

The fact that environmental conditions affect the density and size of fruiting bodies has been appreciated for a long time. Potts (1902) and Harper (1932) showed that light produced more numerous and smaller fruiting bodies. This was confirmed by Raper (1940), who also showed that a slight decrease in humidity

TABLE II

The average radius of the aggregation territories of different species

Species	No. of cases	Mean radius in mm.
D. discoideum	25	1.27
D. purpureum	36	.73
P. violaceum	39	.63
D. mucoroides	24	.53
P. pallidum	31	.49

would elicit the same effect. Bradley, Sussman and Ennis (1956) studied the influence of various chemical agents upon aggregation and they found that histidine in suitable concentrations produced more numerous smaller fruiting bodies, while adenine had the reverse effect. The effect of histidine has been confirmed by recent studies of Krichevsky and Wright (personal communication).

Using the test system described in this paper and *D. purpureum* as the test organism, Heller and Miles (1961) have shown that light is exceedingly effective and that as the intensity increases the aggregation territory becomes correspondingly reduced. They also showed that humidity, in the dark, had a very small effect as compared to the light effect, and that while the aggregation territory became reduced in size with decreasing humidity it soon reached a minimum (very roughly in the neighborhood of 98% relative humidity) and then increased rapidly as the relative humidity of the surrounding atmosphere approached 95%.

In a parallel study Opderbeck (1961) examined the effect of histidine and also successfully repeated the results of the previous workers in the dark, but in the light he found that the histidine had the reverse effect; that is, it produced, in all the concentrations tried (from 10^{-1} to 10^{-3} M), an increase in the size of the aggregation territories as compared to the controls. It is hoped that ultimately further study of these effects (which is in progress) may shed some light on the factors which are responsible for the delineation of the aggregation territory.

Discussion

At the moment the mechanism of territory formation is unknown. It is, of course, possible to suggest many hypotheses, two of which will be mentioned here.

(1) Any cell could become so altered in its state that it would produce an inhibiting substance which diffuses outward and prevents any other cell, within a given radius, from achieving the same state. Clearly the distance the substance diffuses would be independent of the number of cells within a territory. One must also presume that the cell producing the inhibitor lies at the center of the future aggregation pattern, similar to Shaffer's (1961) "founder cell" in *P. violaceum*.

(2) The above hypothesis assumes two separate functions for the keystone cell: inhibition and the subsequent initiation of acrasin production. It might be possible to explain the whole phenomenon solely on the basis of acrasin diffusion. If we assume that the original puffs of acrasin are not carried outward from cell to cell by the Shaffer (1957) relay system, but diffuse from one cell or a small group of cells, and if we assume that a certain concentration of acrasin prevents other cells from becoming acrasin-emitting pace-makers, then this original diffusion gradient of acrasin can be unaffected by the number of cells in a territory and therefore delineate the aggregation territory. Unfortunately, these hypotheses and any other we might invent have far too many assumptions and are in urgent need of testing by experiment.

In relating this work to those of others it first should be mentioned that although Sussman and Noël (1952) and Sussman and Sussman (1961) have made a study of the relation of amoeba density to the number of fruiting bodies, they did not measure either territory size (fruiting body density) or sorocarp size, and therefore it is impossible to compare their work with the present study.

On the other hand, the aggregation territories are obviously related to the problem of the initiation of aggregation and it is pertinent to examine the "initiator cell" hypothesis of Sussman and his group (*e.g.* Sussman and Ennis, 1959; Ennis and Sussman, 1958). This hypothesis assumes that special "initiator cells" are in a fixed proportion to the total cell number: in *D. discoideum* one cell in every 2200 is presumed to be an "initiator cell," while in *D. purpureum* it is one cell in every 300. We have made some cell counts for these two species under threshold conditions where the amoeba density is just sufficient to produce aggregation and fruiting. If the total number of cells per territory is determined (*i.e.*, the number of cells for the sorocarp as well as the number of cells that failed to enter the aggregate) they average 1032 for *D. discoideum* and 150 for *D. purpureum*. In other words there are roughly twice as many centers as there are "initiator cells." However, there are so many other ways of showing that aggregation occurs in small populations of cells below the number predicted from the "initiator cell" hypothesis that the hypothesis may no longer be considered tenable (Bonner, 1960; Konijn and Raper, 1961; Gerisch, 1961).

But perhaps the far more important point is that contrary to the "initiator cell" hypothesis, aggregation is not determined by a cell which holds a strict proportion to the other cells in a population; it is completely independent of the other cells (provided a sufficient cell density is maintained). The only factor which clearly and absolutely controls the initiation process is space: the aggregation territory is, for each species under given environmental conditions, a fixed entity.

These conclusions are entirely in keeping with those of Shaffer (1961), who has shown that existing aggregations in *P. violaceum* are capable of inhibiting the further production of founder cells, even in populations of cells that are not entering streams. According to Samuel (1961) the earliest manifestation of the establishment of the aggregation territory (*i.e.*, initiation) is a regional depression in the rate of cell movement. This is followed, as Shaffer (1961) has shown, by the appearance of a cloud, an area of relatively dense amoebae. In *D. mucoroides* and *D. purpureum* a true aggregation center, with the eventual appearance of incoming streams of amoebae, is only evident after these two initial stages. To completely understand the factors which control initiation and the distribution of the aggregation territory, it will be necessary to provide an explanation for all the events that lead up to the aggregation process itself.

One final point that may have some bearing on future experiments: in previous studies (Bonner, 1960) it was shown that if a small group of cells is isolated by scraping away the cells all around, they often dispersed or disintegrated after aggregation, and in *D. purpureum* the aggregates showed a tendency to produce abnormal sorocarps. In the present study sorocarps of comparable size never disintegrated or showed signs of abnormality. The reasons for this difference are not known but they raise the interesting possibility that if an aggregation territory is isolated and not surrounded by other aggregation patterns, it lacks certain peripheral chemical influences, which results in disintegration or abnormality. The question really is whether or not the cells in neighboring territories remain in communication one with another during aggregation and the later states of morphogenesis.

SUMMARY AND CONCLUSIONS

1. The area of the aggregation territory in the cellular slime molds is constant at different cell densities and therefore the number of amoebae that aggregate in any one territory varies directly with the cell density. As a result sorocarp size in the cellular slime molds is a function of the density of the amoebae prior to aggregation.

2. The mechanism whereby the territory size is determined is not known, although clearly the problem of the initiation of aggregation is related to the establishing of fixed territories. Since their establishment is independent of cell number we may propose the hypothesis that initiation is determined solely by space or distance.

3. There are a number of conditions which frame these general conclusions. The territory size is characteristic for each species and is constant only under a particular set of environmental conditions. Also the relation obviously only applies when the amoeba density is sufficient for aggregation, and each species has a specific range of densities which permit aggregation and fruiting.

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THE GENETICS OF ARTEMIA SALINA.
I. THE REPRODUCTIVE CYCLE[1]

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The brine shrimp *Artemia salina* is a branchiopod crustacean found in saline lakes and the evaporating ponds of commercial salt works. It is of interest to geneticists because amphigonic races have been reported to be diploid ($2n = 42$) or tetraploid. Parthenogenetic races have been found to be diploid, triploid, tetraploid, pentaploid, and octaploid. Barigozzi (1957) has reviewed the cytological studies of these races. No previous attempt has been made to analyze traits which are governed by a single locus.

Artemia is easily cultured in the laboratory because it is resistant to environmental stresses. In a study of a California salt pond containing brine shrimp, Carpelan (1957) found diurnal changes of 12° C. in the water temperature in August. Provasoli and Shiraishi (1959) raised nauplii to adulthood in a sterile medium. Lochhead (1941) reported that females reproduce viviparously or oviparously. The thick-shelled egg contains a blastula and withstands desiccation for as long as 15 years. Therefore, mutant stocks might be conveniently stored in the form of thick-shelled eggs (cysts) without need of repeated subculture.

Because the shrimp is transparent, the effect of genes upon cellular differentiation may be studied throughout the development of one individual. Weisz (1946) has pointed out the advantages of studying morphogenesis in this primitive crustacean which has nineteen body segments but few specialized structures to obscure the principles of development. He has stated (in 1947, p. 87) that the ". . . histological sequences are found to be governed by a continuous overall pattern of metameric development, precisely defined in relative time and in space. . . ."

In 1959, the author began a study of *Artemia* in the hope of developing a method for raising shrimp through many generations in pedigreed cultures. This paper describes a successful culture method and a series of experiments which test for sperm storage by the female and reproduction by parthenogenesis, paedogenesis, and pseudogamy.

MATERIALS AND METHODS

The cysts of the California race were collected from salt works on San Francisco Bay; those of the Utah race were from Great Salt Lake. The dried cysts are routinely stored in glass bottles and hatched in sea water. In 24 to 36 hours, the shells burst and each embryo emerges enclosed within a transparent membrane. In another eight hours, the embryo hatches out as a free-swimming nauplius. The

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nauplii are transferred directly to the *culture medium* made by adding 50 grams of NaCl to one liter of filtered sea water.

All cultures are maintained in 5 cc. of culture medium in shell vials 21 mm. in diameter and 70 mm. high. A *standard yeast suspension* (SYS) is made by mixing 1 cc. of dry Brewer's yeast with 9 cc. of medium. It is dispensed into the shell vials by means of a pipette once per week according to this *standard feeding schedule:*

> *First day.* Nauplii are separated from their parents (in the case of laboratory stocks) or from the shells (if the cysts have been collected from salt ponds). One to three nauplii are put in each vial, 0.05 cc. of SYS is added, and the vial is tightly corked.
>
> *Eighth day.* Again, 0.05 cc. of SYS is added to each vial, irrespective of the number of surviving metanauplii.
>
> *Fifteenth day.* Five-one hundredths cc. of SYS is added for every shrimp present in the vial. Many will have reached sexual maturity. Males are easily identified by their larger antennae.
>
> *Twenty-second day.* Five-one hundredths cc. of SYS is added for every shrimp in the vial. Mated pairs may have produced a brood of nauplii.
>
> *Twenty-ninth day, etc.* The weekly feedings of 0.05 cc. of SYS per adult are continued.

The stocks are maintained at room temperature (20°–28° C.) in a room illuminated during the day by artificial light. No attempt is made to aerate the medium nor to remove waste materials. When raised by this method, more than half of the nauplii born to laboratory stocks reach sexual maturity and a few shrimp have reached the age of nine months. Mated females give birth to free-swimming nauplii; virgin females release transparent thin-shelled eggs which sink to the bottom of the vial and do not hatch. Opaque thick-shelled cysts are not produced unless the culture method is modified.

RESULTS AND DISCUSSION

1. *Effect of food quantity upon viability and fertility*

The standard feeding schedule described above was adopted because shrimp can reach maturity if the standard amount of food is either doubled or cut in half; *i.e.*, it allows margin for measurement errors. It also results in optimum viability, as the following experiment demonstrates. One first-instar California nauplius was placed in each of 276 vials containing 5 cc. of culture medium. All nauplii received the standard amount of SYS until the fifteenth day when the 198 survivors were divided into three equal groups of 66. Each group then was put on a different regimen: 0.025 cc., 0.05 cc., or 0.10 cc. of SYS per shrimp each week. The data in Table I indicate that viability was highest in those shrimp receiving the standard quantity (0.05 cc.) of SYS. Females were examined by transmitted light under a binocular microscope to see if opaque yolk granules were in the eggs. Because vitellogenesis was first seen in females receiving 0.1 cc. of SYS per week (Table II), we may conclude that females fed twice the standard amount mature faster than those on the standard schedule.

Table I

Effect of quantity of food upon viability

Amount of SYS each week	Number of living shrimp Age (days) 15	22	29	36	57
0.025 cc.	66	41	30	24	14
0.05 cc.	66	44	38	34	28
0.10 cc.	66	44	38	35	20

2. *Relation of age and fertility*

When different males were mated successively to a fertile female, none was found to be sterile. But many females consistently produced either small broods of nauplii or broods of thin-shelled eggs which did not hatch. About one-half of both the wild and the mutant females had high fertility records like those shown in Table III. Note that the number of nauplii per brood is not correlated with the age of the female. The brood size may be influenced by uncontrolled factors such as the type of bacterial flora in the vial or the time of feeding in relation to the reproductive cycle. In another experiment, four pairs of shrimp, all of which were over five months of age, produced broods of normal size (39–76 nauplii) and the nauplii had normal viability. Fertility evidently does not decline throughout the first five months of life.

3. *Tests for parthenogenesis and paedogenesis*

To test for parthenogenesis, 20 immature females of the Utah race and 80 of the California race were isolated, one in each vial. They did not produce nauplii but laid broods of transparent eggs every four or five days. A control group was kept for the same period of one month in the presence of males. All of the 20 Utah controls and 64 of the 80 California controls gave birth to nauplii.

More than 200 females of each race have been isolated and parthenogenesis has never been observed. Nauplii are born three to five days after the females have

Table II

Effect of quantity of food upon fertility

Amount of SYS each week	Females showing vitellogenesis/total females Age (days) 22	29	36	57
0.025 cc.	0/20	0/15	0/10	3/5
0.05 cc.	1/22	6/18	11/16	11/12
0.10 cc.	10/22	17/20	16/18	11/12

mated and at no other time. All attempts to hatch the eggs of virgin shrimp have failed. These findings are in agreement with those of Lochhead (1941), who found that fertilization was essential for reproduction in the California race. However, both Jensen (1918) and Relyea (1937) reported that the Utah race could reproduce parthenogenetically. These two authors did not provide sufficiently detailed accounts of their experiments to permit an attempt to replicate them in this present study.

In order to test for paedogenesis, California nauplii were allowed to grow to adulthood in the presence of their mother. In the fourteen cultures observed, the male nauplii were unable to fertilize their mother or their female sibs until they reached the tenth instar of Heath (1924) when their antennae took on the adult shape, enabling them to clasp the femal[illegible] In 24 cultures, California females raised with their fathers also failed to reproduce until they reached adulthood and vitellogenesis was present. Hundreds of nauplii from both races have been paired during routine maintenance of stock cultures, with no evidence of paedogenesis.

TABLE III

Relation of age and fertility in three Utah females

Female	Number of nauplii in brood								
	Age 4–7 weeks				Age 8–12 weeks				
A	9	19	16	31	70	38	30		
B	17	45	15	56	20	19	36	41	70
C	69	3	42	58	71	92	2		

4. *Description of the gene for red eye*

The first red-eyed shrimp was found by Miss Jean Hanson in the fall of 1960, in the progeny of a brother-sister mating in the Utah stock. The data in Table IV indicate that the gene for red eye, *r*, is a recessive and has complete penetrance in the homozygote. Because the reciprocal crosses of the type $RR \times rr$ yield no red progeny, we may conclude that the gene is not sex-linked. Only 138/682, or 20%, of the F_2 shrimp had red eyes. The deviation from the expected 25% is highly significant ($P < 0.01$) and suggests that the red-eyed shrimp may have a lower viability than that of the black-eyed shrimp. The data from the ten backcross ($Rr \times rr$) matings also suggest a lower viability of the red-eye phenotype although the deviation from the expected 1 : 1 ratio is not significant ($P = 0.50–0.30$). Because the backcross data favor a single factor hypothesis, we may conclude that the red eye characteristic is governed by one locus. The three phenotypes may be described in this manner:

RR (black). The ocellus is pale red in the first instar of Heath (1924), black in subsequent instars. The two lateral eyes are black from the time they are first pigmented in the third instar and remain black throughout the life of all wild type shrimp.

Rr (black). The ocellus is pale red in the first instar, black in subsequent instars. The eyes are black throughout the life of most *Rr* shrimp. In rare instances, the eyes turn a deep ruby for a few days in the second week of life but revert to black.

rr (red). The eyes and ocellus are pale red for the first ten days; the pigment is so sparsely distributed throughout the first five instars that the three pigmented areas cannot be seen when the metanauplius is examined under the binocular microscope (7 ×). At the end of the second week, the eyes and ocellus are bright red. The eyes darken progressively after the shrimp has reached sexual maturity; by the twenty-second day, the eyes are dark ruby or black. The ocellus remains red for a longer period but may also turn ruby or black.

5. *Test for pseudogamy*

Pseudogamy is defined as the development of an egg parthenogenetically after the initial stimulus of penetration by a sperm. (The sperm nucleus then degenerates and has no effect on the genotype of the offspring.) Because the 39 matings of the type ♂*RR* × ♀*rr* (listed in Table IV) produced only black-eyed offspring, we may conclude that pseudogamy is not the normal form of reproduction in Utah shrimp reared under standard laboratory conditions.

TABLE IV

Segregation of the gene r *in the Utah race*

Number of matings	Mating	Progeny		
		Black	Red	Total
56	*rr* × *rr*	0	1793	1793
10	*Rr* × *rr*	161	149	310
32	*Rr* × *Rr*	544	138	682
39	♂*RR* × ♀*rr*	720	0	720
12	♂*rr* × ♀*RR*	236	0	236

6. *The sequence of events in the reproductive cycle*

The reproductive system of the female consists of two ovaries, two pouch-like oviducts, and a ventral median uterus. The following events normally take place in a 24- to 48-hour period. The female expels from the uterus the first egg generation (brood A) as either virgin eggs or nauplii. The birth process takes from two to ten hours. She molts in a few seconds and then the next egg generation (brood B) passes from the ovaries into the oviducts in less than two hours. They remain there from one to 40 hours, whether copulation occurs or not. They then pass into the uterus, the process taking less than 30 minutes.

The eggs remain in the uterus for three to five days, irrespective of whether or not they are fertilized. The cycle is normally completed in from four to six days. However, in three exceptional females, the eggs lodged in the oviducts for ten days and the cycle was prolonged.

Lochhead (1941) correctly stated this sequence of events but did not publish the evidence for his conclusion that copulation occurred when the eggs were in the oviducts. Therefore, nine *rr* females were successively mated to males of *rr, RR,* and *rr* genotypes but the *RR* male was present only at the time when the eggs were seen to be in the oviducts. The *RR* males often failed to clasp during this short period and the females then laid eggs. Nauplii were obtained from three females; observations on one of them are in Table V. In all three cases, the *RR* male was present only during the time when the eggs were in the oviducts yet all the progeny were of the *Rr* genotype. Fautrez-Firlefyn (1957) and Goldschmidt (1952) have reported that eggs in the oviducts are in metaphase of the first meiotic division.

TABLE V

Observations on the reproductive cycle of one rr *female*

Duration of period	Events
4 days	The first male (*rr*) clasps the female. Egg generation A undergoes segmentation in the uterus. Egg generation B becomes visible in the ovaries due to accumulation of opaque yolk.
10 hours	70 nauplii (brood A) are expelled from the uterus. The *rr* male continues to clasp and attempts unsuccessfully to copulate. The female molts. The clasping pair is transferred to a slide and the male is pulled away. The female is returned to the vial.
55 minutes	Egg generation B passes into the oviducts. The second male (*RR*) is added. He clasps and copulates. Afterward, one seminal vesicle is transparent; the other is opaque due to the presence of sperm. The clasping pair is transferred to a slide and the male pulled away. The female is returned to the vial. Egg generation B passes into the uterus.
4 days	The third male (*rr*) is added. He clasps the female within twenty minutes after the eggs have entered the uterus. Four days later, 115 nauplii (brood B) are born.

58 nauplii of brood A survive to an age when they can be classified. All have red eyes. The 98 survivors of brood B have black eyes.

7. *Studies of the female reproductive cycle with tests for sperm storage*

Observations on more than 200 females of each race indicated that if they mated once, they produced a single brood of nauplii and thereafter laid thin-shelled eggs which did not hatch. This suggests that *Artemia* females do not store sperm as do *Drosophila* females. However, this evidence is not conclusive because the acts of clasping or copulation might in some way be essential for egg maturation. (For example, copulation might be the stimulus needed to bring about the reflex secretion by the oviduct of a substance which would cause the eggs to complete the first meiotic division.) This possibility is remote but, if true, it would invalidate the previously described tests for parthenogenesis as well as those for sperm storage. Therefore, the following experiments were designed to rule out this

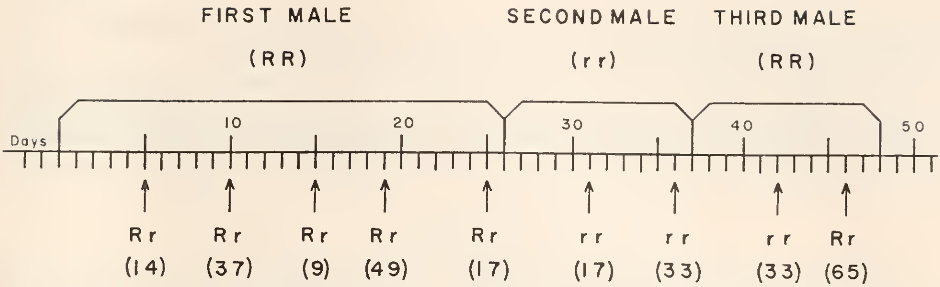

FIGURE 1. Genotypes of nine successive broods of nauplii produced by one *rr* Utah female mated to three different males. The numbers in parentheses indicate the number of progeny in each brood which survived to an age when their eye color could be classified.

possibility and to test for parthenogenesis and for sperm storage in a female which was at all times in the presence of a male.

RR males were hatched from cysts collected from Great Salt Lake; *rr* shrimp were selected from the Utah laboratory cultures. Twenty *rr* females were studied; each was alternately mated to males of *RR* and *rr* genotypes. The record of one of these females is seen in Figure 1. On the twenty-fifth day, a brood of *Rr* nauplii was produced as the result of a mating with an *RR* male. On the twenty-sixth day, the first male was removed, a second male with *rr* genotype was added, the eggs moved into the oviducts, and copulation occurred. These observations on the transparent female are confirmed by the fact that the nauplii born on the thirty-first day had the *rr* genotype. On the thirty-sixth day a brood of *rr* nauplii was born, the next generation of eggs passed into the oviducts, and the female again mated with the second male. On the thirty-seventh day, a third male was added, he attempted to copulate, but was unable to affect the genotype of the next brood because the eggs were now in the uterus.

In Figure 1, two changes in brood paternity may be seen: one between the twenty-fifth and thirty-first days and another between the forty-second and forty-sixth days. Another six changes in brood paternity are summarized in Table VI; in each case the two broods (A and B) are less than six days apart. Note that in the second (B) brood, all the nauplii have the same genotype because sperm are not stored by the female from one cycle to the next. Corroborative data were obtained from the other females, but in each case a brood of virgin eggs

TABLE VI

Comparison of pairs of broods of different paternity born to six rr *females*

Brood	Number of nauplii classified and their genotype					
	Female					
	1	2	3	4	5	6
A	73 *Rr*	30 *rr*	31 *Rr*	58 *Rr*	17 *rr*	22 *rr*
B	56 *rr*	46 *Rr*	41 *rr*	33 *rr*	26 *Rr*	12 *Rr*

separated the two broods of different paternity because the second male failed to clasp in the short period when the eggs were in the oviducts.

The author wishes to express her gratitude to three students who subcultured the stocks used in this study: Carol Cleminshaw, Jean Hanson, and John Parker. Thanks are due to Dr. John S. Hensill, who suggested the use of *Artemia* in genetic experiments, and made many valuable suggestions during this investigation.

SUMMARY

1. This paper reports the first analysis of an inherited trait governed by one locus in the brine shrimp, *Artemia salina*. The autosomal gene, *r*, for red eyes arose spontaneously in a Utah race. It is recessive to the wild type allele, *R*, for black eyes. It has complete penetrance in *rr* shrimp.

2. The standard culture method outlined here has successfully carried the mutant stock through ten generations in a one-year period.

3. Reproduction was studied in two races from California and Utah. Neither paedogenesis nor parthenogenesis was observed in these shrimp which were raised by the standard culture method. This observation conflicts with the reports of Jensen and of Relyea that the Utah race could reproduce parthenogenetically.

4. Matings of *RR* males to *rr* females produce only black-eyed progeny. This indicates that when raised by the standard method the Utah shrimp do not normally reproduce by pseudogamy.

5. Studies of the sequence of steps in the female reproductive cycle confirm the observations of Lochhead. Genetic experiments have demonstrated that although the adults may clasp continuously throughout the cycle, copulation is effective only when the eggs are in the oviducts.

6. Females do not store sperm from one reproductive cycle to the next. If an *rr* female is alternately mated in different cycles to males of *RR* and *rr* genotype, all the nauplii in one brood have the same genotype.

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SURVIVAL AND GROWTH OF LARVAE OF THE EUROPEAN OYSTER, O. EDULIS, AT LOWERED SALINITIES

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The European oyster, *Ostrea edulis*, in its native habitat, is found primarily in oceanic or nearly oceanic salinities. Its native range extends from the southern coast of England and the Scandinavian countries to the Mediterranean, but recently it has been successfully introduced into New England waters (Loosanoff, 1951, 1952, 1955; Welch, in press).

Initially, Loosanoff (1955) proposed this oyster for introduction into those areas where the summer water temperatures rarely, if ever, are high enough for American oysters to reproduce. More recently, because of heavy mortalities of American oysters in several states, he has suggested that *O. edulis* might be introduced as a second commercial oyster into these and other oyster-producing areas of the United States. Since European and American oysters belong to different genera, they will not interbreed and, what could be extremely important, European oysters might not be susceptible to some of the diseases now affecting American oysters. Moreover, since European oysters are larviparous and their larvae are usually 175 μ to 185 μ at the time of release, the food requirements of the larvae are not as restricted as those of the young larvae of American oysters. Therefore, good sets of European oysters might frequently be obtained in seasons when setting of American oysters fails.

Korringa (1941) reviewed the literature on the effects of salinity on eggs and larvae of several species of oysters, including *O. edulis*. He found no correlation between rate of growth of larvae or intensity of their setting and differences in average salinities in the Oosterschelde, Holland, ranging from 25 to 35 ppt. He quotes the assumption of Gaarder (1932, 1933) and Gaarder and Bjerkan (1934) that 24 ppt is the lowest salinity for satisfactory growth of larvae of *O. edulis*. Korringa also states that changes of salinity, within the range found in the Oosterschelde, cannot be held responsible for the success or failure of spatfall of *O. edulis* in this area, and points out that experiments *in vitro* had not yet been carried out to determine the effect of salinity upon setting.

Walne (1956) reported experiments on rearing *O. edulis* larvae in salinities initially adjusted to 21.1, 25.9, 27.9 and 31.3 ppt. The water in the larval cultures was not changed during the course of his experiments and, apparently due to evaporation and the addition of algal food suspension, the salinity in all cultures increased. Thus, in cultures initially at 21.1 ppt the salinity increased to 25.9 and 26.2 ppt. Under these conditions he found that the larvae survived and grew throughout the salinity range tested, but he did not obtain any spatfall in the cultures started at 21.1 ppt.

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The present study was undertaken to obtain more precise data on the effect of low salinities on egg production and incubation in *O. edulis,* upon survival and growth of larvae, and upon setting of mature larvae. Since several of the oyster-producing areas in the United States, where we might wish to introduce *O. edulis,* are characterized by relatively low salinities, such information is necessary.

METHODS

The methods employed were essentially the same as those used by Davis (1958) in a similar study of the salinity tolerance of eggs and larvae of the American oyster. However, since *O. edulis* is larviparous, to obtain larvae from these oysters it was necessary to hold adults in aquaria during the period of gonad development, spawning and incubation. Approximately ten adult oysters were kept in each aquarium in 60 liters of water. One group of oysters was kept at our normal salinity (27 ppt); another group was kept at a salinity of 20 ppt; and a third group was kept at 17.5 ppt. We used sea water, filtered through an Orlon filter designed to remove particles above 15 μ in diameter, and lowered the salinity to the desired level with demineralized tap water. Water in these aquaria was changed daily and three to four liters of algae were added during each change to supplement the food present in the water.

The adult oysters kept at normal salinity provided the larvae used in these experiments. As soon as larvae were noticed after release, they were collected by draining the water from the aquarium through a 250-mesh screen. By using care to drain without disturbing the sediment on the bottom of the aquarium, healthy larvae were collected relatively free of debris. These larvae were then resuspended in a three-liter Pyrex jar, the number of larvae per ml. determined, an appropriate volume pipetted into each of a series of one-liter polyethylene beakers, and the volume made up to one liter of the desired salinity. Two beakers of larvae were set up at each of the following salinities: 27 ppt (control), 25 ppt, 22.5 ppt, 20 ppt, 17.5 ppt, 15 ppt, 12.5 ppt and 10 ppt.

In the first experiment we used 6400 and in the second, approximately 13,000 larvae per beaker. Following the procedure of Davis (1958), to hold salinities constant, the one-liter cultures received food only every second day when the water was changed, instead of daily as is the usual practice. All the cultures were covered to prevent excessive evaporation and kept in a constant temperature bath at 23° C. ± 1° C.

In the two experiments to determine the effect of low salinities on setting of mature larvae, we reared the larvae in 15-liter culture vessels at 27 ppt until their average size was 250 μ and many were already in the 275 to 300 μ range. Approximately 9000 of these larvae were pipetted into each of a series of one-liter beakers filled with water adjusted to the desired salinities. In the first experiment, because of the limited number of mature larvae available, we used a single culture at each salinity, but in the second, mature larvae were abundant and duplicate cultures were used at each salinity.

A single oyster shell in each beaker was used as cultch. These shells were replaced every second day, as the water was changed, and the shells removed were examined under a dissecting microscope to determine the number of spat caught. Only those setting on the smooth, white, inner surface of the shell were counted. Since many oysters also set on the rough, dark, outer surface of the shell, on the walls of the container, and on small shell fragments or other debris where it is impossible to count, the number of spat recorded is only a rough index of the total number setting.

RESULTS

Effects of lowered salinities on growth of larvae

The results of the first experiment showed that growth of larvae was virtually normal in salinities as low as 22.5 ppt (Fig. 1). At 20 ppt, although larvae grew

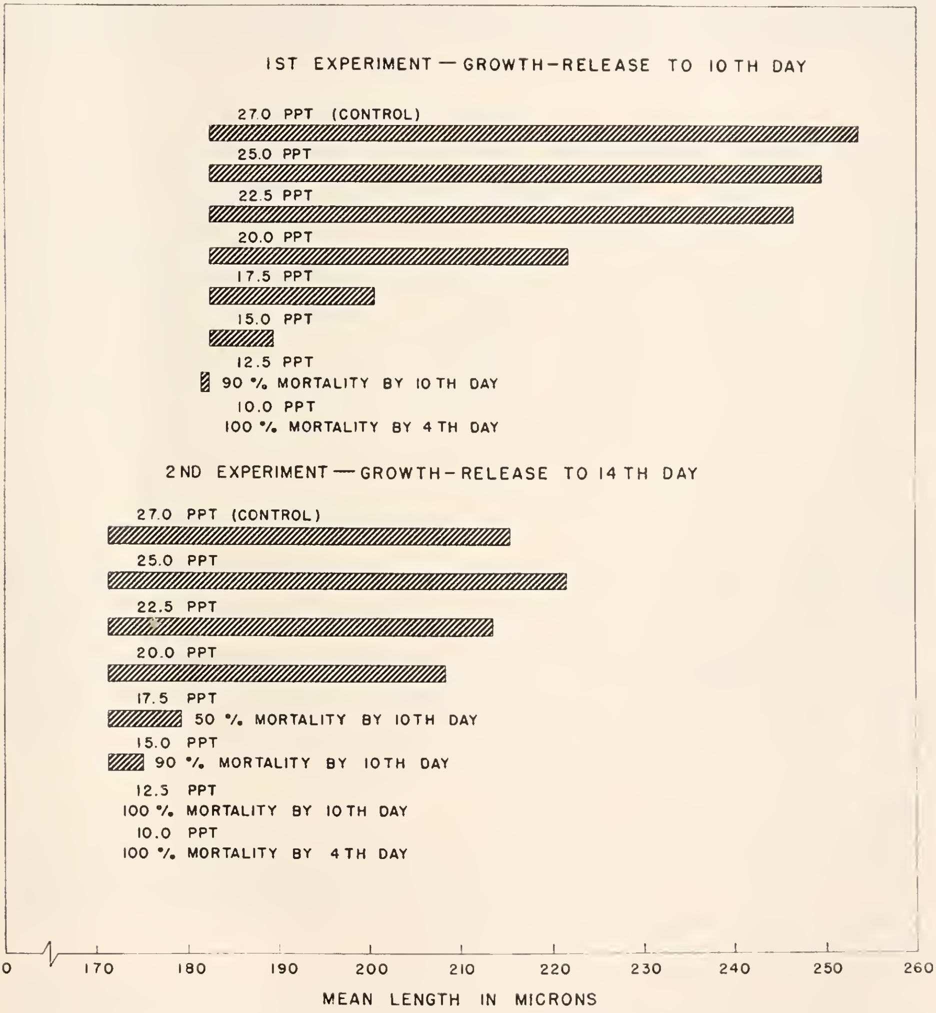

FIGURE 1. Growth of *O. edulis* larvae at different salinities. Mean lengths are based on measurements of 100 larvae from each of duplicate cultures at each salinity.

at a considerably slower rate, some were reared to setting stage and metamorphosed. At 17.5 ppt growth was extremely slow; no larvae reached the setting stage and eventually all died. At 15 and 12.5 ppt, growth of larvae was even slower, but some lived at each salinity for ten days or more. Larvae kept at 10 ppt all died within four days.

The larvae used in the second experiment were about 11 μ smaller at the time of release and grew at a considerably slower rate than those used in the first experiment (Fig. 1). Since larvae from the brood used in this second experiment grew normally in other cultures set up at the same time, we believe the slower growth was the result of the higher concentration of larvae (13,000 per liter as opposed to 6000 per liter in the first experiment). This would indicate that to assure good growth these larvae must be kept in much lower concentrations than larvae of either *Venus mercenaria* or *Crassostrea virginica*, both of which grow quite well at 13,000 individuals per liter.

TABLE I

Effect of reduced salinities on setting of larvae reared almost to setting stage at 26–27 ppt

Salinity in parts per thousand	Average number of spat per culture	
	1st experiment	2nd experiment
26–27 (Control)	147	134
25.0	504	145
22.5	120	171
20.0	114	38
17.5	24	5
15.0	4	0.5
12.5	0	0
10.0	0	0
7.5	0	0

The pattern of growth was generally the same in both experiments, even though growth of larvae was much slower in the second experiment. Growth of larvae was not greatly different from that in control cultures at salinities of 20 ppt and higher. As in the first experiment, the rate of growth dropped off sharply between 20 ppt and 17.5 ppt. Mortality was high and growth negligible at all lower salinities. Because of the very slow growth of larvae, the second experiment was discontinued before larvae in any of the cultures reached setting stage.

Effects of lowered salinities on setting

Two experiments were run to determine the minimum salinity at which larvae of *O. edulis* could set. In both, larvae that had been reared almost to metamorphosis at our normal salinity of 26–27 ppt were transferred directly to lowered salinities and their setting recorded (Table I).

Although a salinity of 20 ppt was the lowest at which larvae could be reared from release through metamorphosis, some mature larvae transferred to salinities of 17.5 and 15 ppt did set (Table I).

It is significant that all of the set obtained at 17.5 and 15 ppt occurred within four days after the larvae were transferred to these salinities, while setting continued for as long as 14 days in higher salinities. This indicates that only those larvae that were almost ready to set at the time of transfer were able to complete growth and metamorphose at these lower salinities, while all less developed larvae died. However, even those larvae that were ready to set at the time of transfer were unable to complete the process of metamorphosis at salinities of 12.5 ppt or lower.

A similar record of the number of set obtained from larvae reared at lowered salinities showed that while there was no significant mortality within ten days in a salinity of 15 ppt nor in any of the higher salinities, none of the larvae grown at 15 and 17.5 ppt survived to set. Even in cultures kept at 20 and 22.5 ppt, significantly fewer larvae succeeded in completing metamorphosis than in cultures reared at 25 ppt or in control cultures.

Effects of lower salinities on gonad development, spawning and incubation

Because it was found (Davis, 1958) that eggs from *C. virginica* that had developed gonads at salinities of about 8.75 would develop into straight hinge larvae at considerably lower salinities than would eggs from parents that had developed gonads at 26–27 ppt, we thought it worth while to attempt to induce gonad development, spawning and incubation of *O. edulis* at lowered salinities. We hoped to use larvae from oysters kept at salinities of 20 ppt and 17.5 ppt in salinity experiments parallel with those on larvae from oysters kept at our normal salinity, to determine whether the salinity tolerances of the larvae were altered by the salinity in which the parent oysters had developed gonads and spawned.

The adult *O. edulis* that were kept in aquaria at 20 ppt and at 17.5 ppt failed to release any normal living larvae. In other respects, however, transfer to these lowered salinities appeared to affect them for only a short time. Oysters transferred to 17.5 ppt, for example, failed to feed normally for only a few days, but thereafter cleared the water of the algae added as food, as did those kept at higher salinities. These oysters were kept in these salinities for 60 days and appeared normal in every respect, except that they produced no normal larvae.

Evidence of spawning of at least one female, in the group kept at 20 ppt, was observed, but no living larvae were recovered. A few empty larval shells were found on several occasions, but there were not enough of these to account for a normal brood from a single female. Although spawning was not observed in the group kept at 17.5 ppt, on several occasions highly abnormal living larvae were recovered. These larvae either had no shells at all or had small, highly abnormal ones. Nevertheless, many of them were capable of taking food and lived for several days after transfer to normal sea water. Because there were only a few of them, their further development could not be followed.

DISCUSSION

The results of our experiments indicate that culturing of *O. edulis*, in areas where the salinity is 20 ppt or lower, cannot be successful because these salinities are too low for reproduction of this species. It is possible, nevertheless, that in situations where there is a gradation of salinities, mature larvae, developing and growing to setting size at salinities of about 22.5 ppt or higher, could be carried by currents to salinities as low as 15 ppt and set and grow there.

Since some of the larvae reared at 20 ppt did metamorphose, it is possible that culture of *O. edulis* at this salinity might be successful, but growth of larvae would be slow and the intensity of setting reduced. Moreover, adult oysters kept at this salinity failed to give any living larvae. While it is possible that we might have had different results if we had used more oysters, the number of oysters (only 10) kept at this salinity was the same as the number kept at our normal salinity that released the several million larvae used in these experiments.

The release of abnormal veligers by oysters kept at 17.5 ppt is, we believe, positive evidence that this salinity is too low for normal larval development. It is possible that oysters acclimated to this salinity over a longer period of time might have given viable larvae. However, the results of Davis (1958) showed that keeping adult American oysters, which had developed gonads at 8.75 ppt, for only a few days at salinities of 7.5, 10 and 15 ppt altered the salinity tolerance of their eggs.

Previous investigators using, for the most part, field data have shown that *O. edulis* larvae can grow and set at salinities as high as 34 to 39.5 ppt (Mazzarelli, 1924). Korringa (1941) also states that, "It is my opinion that variations in salinity between 25 ppt and 35 ppt probably have little or no influence on larval growth and development in *Ostrea edulis*" (pp. 133–134).

Our results cannot be compared directly with those of Walne (1956) because he did not change the water and his salinities were not held constant. Using our methods, however, larvae were reared to metamorphosis and some set was obtained at salinities of 20 and 22.5 ppt, both lower than the lowest initial salinity (25.9) from which Walne obtained spatfall.

Our results confirm Korringa's opinion that a salinity of 25 ppt has no appreciable adverse effect on growth of larvae. They further show that the lower limit for good growth and setting is about 22.5 ppt, although larvae can grow to metamorphosis at 20 ppt and mature larvae can set at even lower salinities.

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SUMMARY

1. At salinities of 25 and 22.5 ppt growth of larvae of *O. edulis* and intensity of setting was not significantly different from that in control cultures at our normal salinity of 26–27 ppt.

2. At 20 ppt growth of larvae was appreciably slower than at higher salinities and the intensity of setting was reduced.

3. At 17.5 and 15 ppt larvae lived for some time and showed appreciable growth, but they all died prior to metamorphosis.
4. At 12.5 ppt larvae showed no growth and by ten days after swarming they had suffered 90% or higher mortality.
5. At 10 ppt all larvae died in less than four days.
6. Some larvae that had been reared to setting size at a salinity of 26–27 ppt were capable of setting in salinities as low as 15 ppt.
7. No normal larvae were obtained from adult oysters kept at salinities of 20 or 17.5 ppt.

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THE BIOLOGY OF ASCIDIA NIGRA (SAVIGNY). I. SURVIVAL AND MORTALITY IN AN ADULT POPULATION

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Ascidia nigra is a solitary ascidian with a jet black test and measuring 10 to 12 cm. in length when fully grown. The colour of the test is due to pigment granules which migrate out through the test and form a thin layer at the surface where the pigment continuously sloughs off, together with some of the test substance. This process, which was first described by Hecht (1918) and has been confirmed by the writer, makes it nearly impossible for other sedentary organisms to settle and grow on the test. Unlike many other ascidians, therefore, *A. nigra* has a clean test throughout its life and this, combined with its conspicuous colouration, makes it an ideal animal for ecological study; against its background it stands out conspicuously and the history of individuals may be recorded easily.

The species is widely distributed throughout the warm waters of the western Atlantic ranging from Florida (25° N.) to Saõ Sebastian, Brazil (24° S.). It occurs in Bermuda (32° N.) (Van Name, 1945) and is recorded from the Red Sea, Gulf of Aden and Gulf of Guinea (Millar, 1958). Throughout the West Indies it is one of the commonest inshore shallow-water ascidians, usually confined to the sheltered waters of harbours and mangrove lagoons; it is only rarely found on coral reefs. In the harbours and lagoons it is found attached to mangrove roots, piers, pilings, buoys and ship bottoms from surface level to about 25 feet; it also occurs sometimes on the sea floor where a suitable hard substratum, such as a stone, enables it to settle. In general it is a primary coloniser, developing large populations on new or cleaned surfaces but ultimately being replaced by other organisms. The primary sessile community in Kingston Harbour, Jamaica, is dominated by algae (*Enteromorpha*), cirripedes (*Balanus amphitrite* Darwin), hydroids, serpulids and the ascidians[1] *A. nigra, Didemnum candidum, Diplosoma macdonaldi, Symplegma viride* and *Polyclinum constellatum*. This gives way by various stages to a climax community dominated by sponges (principally *Haliclona* sp., *Tedania ignis* (Duchassaing and Michellott) and *Mycale cecilia* deLaubenfels), anemones (*Aiptasia tagetes* (Duchassaing and Michellott)), ophiurans (*Ophiothrix angulata* Say) and the ascidians *Microcosmus exasperatus, Herdmania momus* and *Pyura vittata,* but in which *Ascidia nigra* is relatively rare. A detailed account of succession in this community will be published in a latter paper.

The present paper describes the history of a population of *Ascidia nigra* which settled and developed naturally on panels suspended in the sea at Port Royal in Kingston Harbour, Jamaica (Station C in Goodbody, 1961a). During the period of observation the population suffered a density-independent mortality following an influx of fresh water to the harbour; the details of this are discussed below and in the paper referred to above.

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[1] Nomenclature of ascidians is taken from Van Name (1945).

METHODS

A raft (or pontoon) four feet square was moored 20 feet away from the sea wall at Morgan's Harbour, Port Royal, where the water is 23 feet deep. On each of the four sides of the raft two panels were suspended one below the other on galvanised chains so that the upper panel had its upper edge 4 feet below the water surface and the lower one 7.5 feet below the surface. The panels hung so that the settling surfaces were vertical in the water. In this way 8 panels were suspended from the raft, 4 at each vertical level.

Each panel was made of a rectangular sheet of ⅛-inch-thick "Tufnol"[2] 18 × 15 inches, bound along each side of each long edge by a thin strip of steel 1.5 inches wide. This reduced the area of "Tufnol" available for settlement to 18 × 12 inches. Both sides of each panel were available for settlement and were designated Front (F) and Rear (R) according to whether it faced out from the raft (F) or in towards the mooring chain (R).

The raft was moored by a single central chain and swivel so that it could turn horizontally about a central axis. Though undesirable from some points of view, this and the small size of the raft were essential parts of an attempt to minimise the risk of destruction by hurricanes.

The 8 panels were placed in position on August 1, 1957, and were not finally removed until May 6, 1960. At intervals the panels were removed from the raft and taken to a tank in the marine laboratory. The maximum time any panel was out of the water at any one time was one minute. In the laboratory the position of each specimen of *A. nigra* was plotted on a map of the panel and a colour photograph of each side of the panel was taken. From the photographs permanent maps were made and each animal designated by a serial number. In this way a permanent record was obtained of the history of each animal whose position was known on the map from its first appearance to its ultimate death or disappearance.

In the course of lifting and examining panels a total of five animals were accidentally killed. These have been excluded from the analysis of data except in Table II which is concerned solely with new settlements.

COLONISATION AND POPULATION GROWTH

Table I shows a summary of the history of the population, and in columns 4 and 5 and in Figures 1 and 2 are shown the number of new animals appearing on the panels and the total population present. It will be seen that the majority of animals colonised before September 26, 1957 (57 days after the start of the experiment) and very few animals colonised after October 25, 1957. Newly settled *A. nigra* do not begin to develop the black pigment until about 20 days after metamorphosis and hence are only just visible to the naked eye as small black

[2] "Tufnol" is the trade name for a synthetic resin bonded fabric sheet conforming to British Standard No. 972: 1941. It is ideal material for experimental panels as it is the colour of dark wood and is completely unaffected by sea water or by boring animals as *Teredo* or *Limnoria*.

objects when 4 weeks old (unpublished observations). It is probable, therefore, that the majority of animals settled on the panels in the first 4 weeks but were not all visible when they were first examined on August 28, 1957. It is clear from these figures that once the primary colonisation is complete few new animals settle in the community. Elsewhere (Goodbody, 1961c) I have shown that *Ascidia nigra* breeds throughout the year in Jamaica, so that the fall-off in numbers of new animals appearing on the panels cannot be due to a seasonal drop in the number of larvae available. The fall-off in numbers must therefore be due to competition, either inter- or intraspecific, most probably the former. If intraspecific competition was important in determining settlement and growth of new individuals it would be expected that late settlers would tend to appear on panels with few other *A. nigra*.

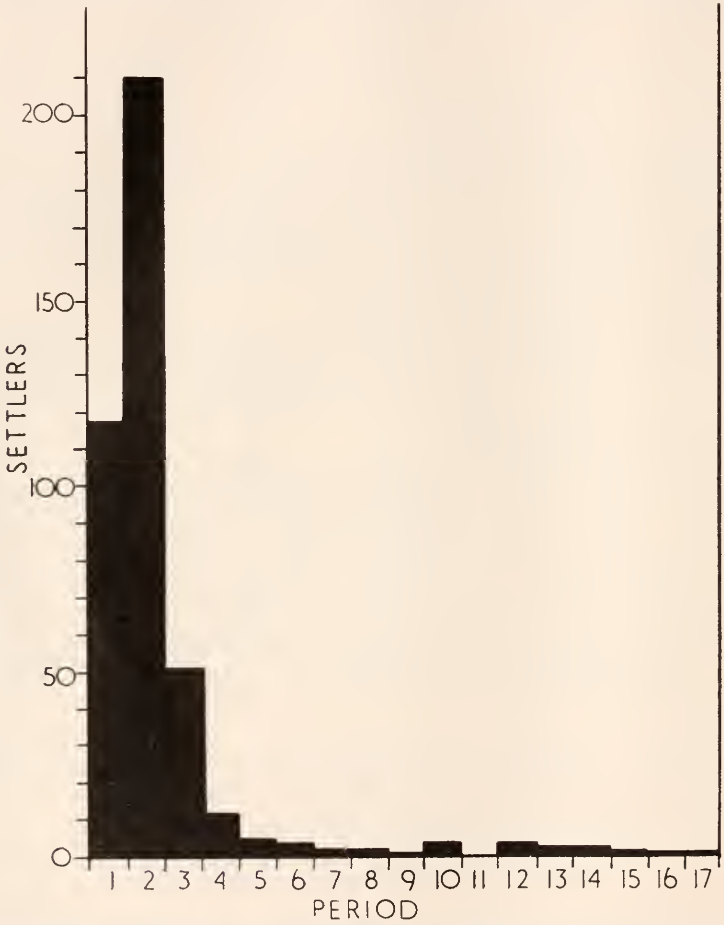

FIGURE 1. The number of new *Ascidia nigra* appearing on panels (settlers) at each inspection throughout the life of the population. The length of each period is not the same (see Table I).

Table II examines this possibility and analyses data for 27 new settlements between November 1, 1957, and October 3, 1958. It is clear from this table that there was no tendency for new settlements to occur on panels with few, as opposed to many, other *A. nigra*. It is more probable that the suppression of later colonisations is an effect of the whole community, in fact a combination of inter- and intraspecific competition. Most of the organisms on the panel are leptopel feeders competing for similar food to that of *A. nigra,* and any newcomer must have difficulty in obtaining sufficient food for growth. Furthermore, sponges may actually inhibit the development of other sessile organisms (Goodbody, 1961b).

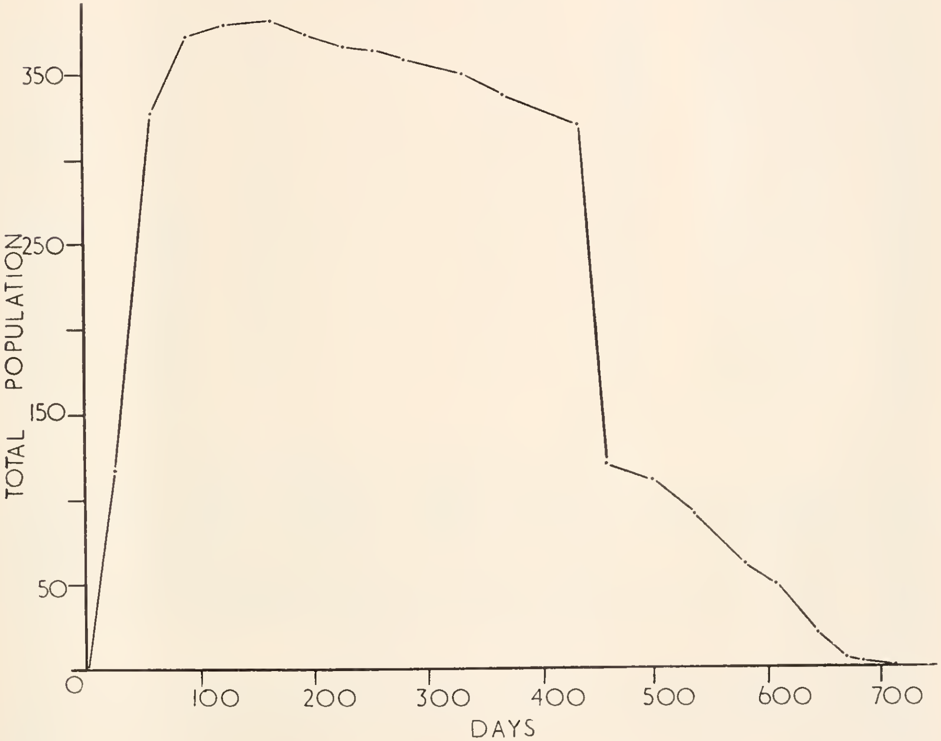

FIGURE 2. The total population of *Ascidia nigra* present on panels throughout the life of the population. The sharp drop after day 435 is due to fresh-water floods (see text).

Figure 2, which illustrates the growth of the total population, also illustrates the rapid rate of colonisation of the new panel. Subsequent to the initial rapid rise in population size, the population becomes stable for a long period, and since few new colonisations are taking place this indicates also a low mortality rate which is discussed in the next section.

The data outlined above, depicting *A. nigra* as a primary coloniser in sessile communities, are substantiated by numerous field observations of natural situations. In recent years several mass mortalities have occurred among sessile communities in Kingston Harbour (Goodbody, 1961a) and subsequently large areas of cleaned

Table I

Total settlement, mortality and mortality index. Mortality index is found by multiplying percentage mortality by $\frac{100}{\text{no. days}}$

Period	Date	Interval in days	Total population	No. new animals settling	No. animals dying	% Mortality	Mortality index
1.	28. 8. 57	28	117	117	—	—	—
2.	26. 9. 57	29	327	210	0	0	0
3.	25. 10. 57	29	372	50	5	1.5	5
4.	27. 11. 57	33	379	11	4	1.1	3
5.	8. 1. 58	42	381	4	2	0.5	1
6.	6. 2. 58	29	373	3	11	2.9	10
7.	12. 3. 58	34	366	1	8	2.1	6
8.	9. 4. 58	28	363	1	4	1.1	4
9.	6. 5. 58	27	358	0	5	1.4	5
10.	25. 6. 58	50	350	3	11	3.1	6
11.	30. 7. 58	35	337	0	13	3.7	11
12.	3. 10. 58	65	320	3	20	5.9	9
13.	27. 10. 58	24	120	2	202	63.1	263
14.	8. 12. 58	42	110	2	12	10.0	24
15.	13. 1. 59	36	90	1	21	19.1	53
16.	26. 2. 59	44	60	0	30	33.3	76
17.	26. 3. 59	28	49	0	11	18.3	65
18.	30. 4. 59	35	19	—	30	61.2	175
19.	27. 5. 59	27	4	—	15	78.9	292
20.	10. 6. 59	14	2	—	2	50.0	357
21.	7. 7. 59	27	0	—	2	100	370

surfaces became available for larval settlement on mangrove roots, piers, pilings, etc. *A. nigra* has always been one of the first colonisers of these surfaces, sometimes developing as dense clusters of several hundred individuals. Similarly, whenever new piles are driven this species develops in dense clusters in the early stages. However, as the sessile community develops on these surfaces, *A. nigra* is slowly replaced by dominants such as *M. exasperatus*, *P. vittata* and *H. momus*, together with sponges, anemones and lamellibranchs.

Why *A. nigra* should succeed only as a primary coloniser is not clear at present, but it is pertinent to point out that at all stages of its growth the siphons project

Table II

Settlement of new animals, in relation to density of A. nigra *already on panel, between 25th October 1957 and 3rd October 1958*

Panel density	No. of panels	No. of settlements	No. of settlements per panel
11–15	4	6	1.5
16–20	2	2	1.0
21–25	4	6	1.5
26–30	3	7	2.3
31–35	2	5	2.5
36–40	1	1	1

out into the water beyond the level of the remainder of the community, including other ascidians. This suggests that competition for food may be of paramount importance and that by keeping the siphons projecting it can tap the food supply before it reaches the other members of the community.

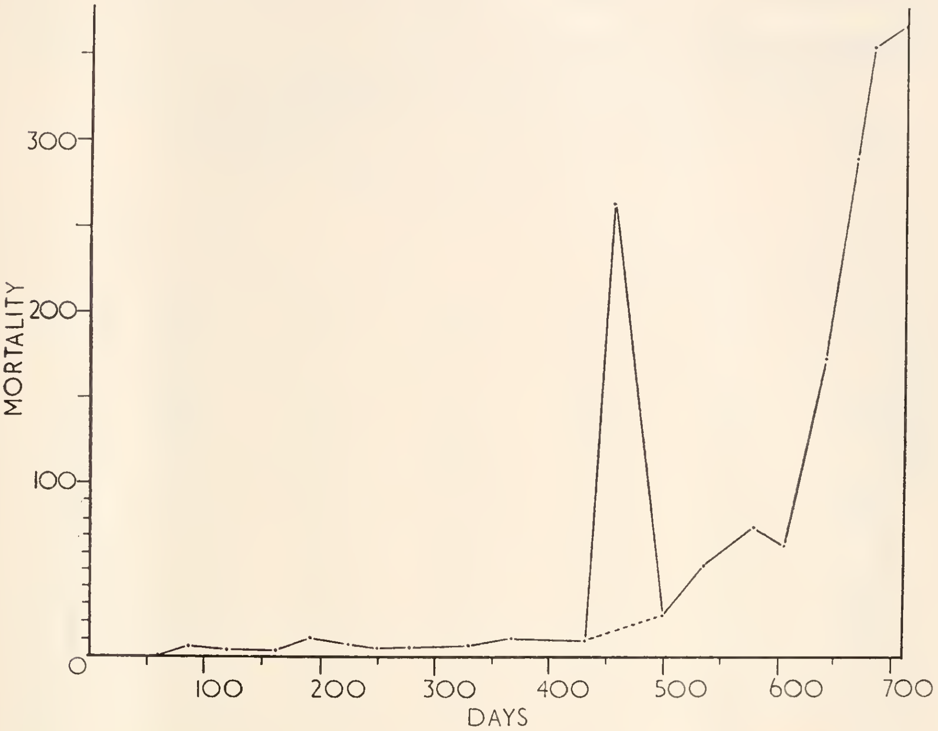

FIGURE 3. Mortality rate expressed as mortality index (see text). The dotted line connects the two periods before and after the fresh-water floods.

MORTALITY AND SURVIVAL

The intervals between successive inspections of the panels were not constant and so it is not possible to construct the usual form of life table. The succeeding analysis is therefore confined to two functions of the life table, mortality rates and survival. In place of the usual "mortality rate" (1000 qx) I have substituted a "Mortality Index" which may be expressed as

$$\frac{100}{I} \times \frac{100D}{P} = (I \times 10^{-1})\ 1000\ qx,$$

where I is the interval in days, D is the number of deaths during the interval and P the total population at the beginning of the interval. The Mortality Index provides a means of comparing the mortality rate over the whole life span of the population.

The mortality index for the whole population is shown in Table I and Figure 3. In calculating this function no allowance has been made for the small increment in the population subsequent to the establishment of the main population. It is clearly demonstrated here that from the time animals first appeared as small black ascidians, the mortality rate was low until the population was about 15 months old, after which it climbed rapidly until the last animal died when the population was 23 months old. The greatest life span attained by any one animal was an individual which appeared at the first inspection, hence settled in the first week of August, 1957, and died between May 27 and June 3, 1959. It must therefore have been between 94 and 96 weeks old (22 months) at the time of death.

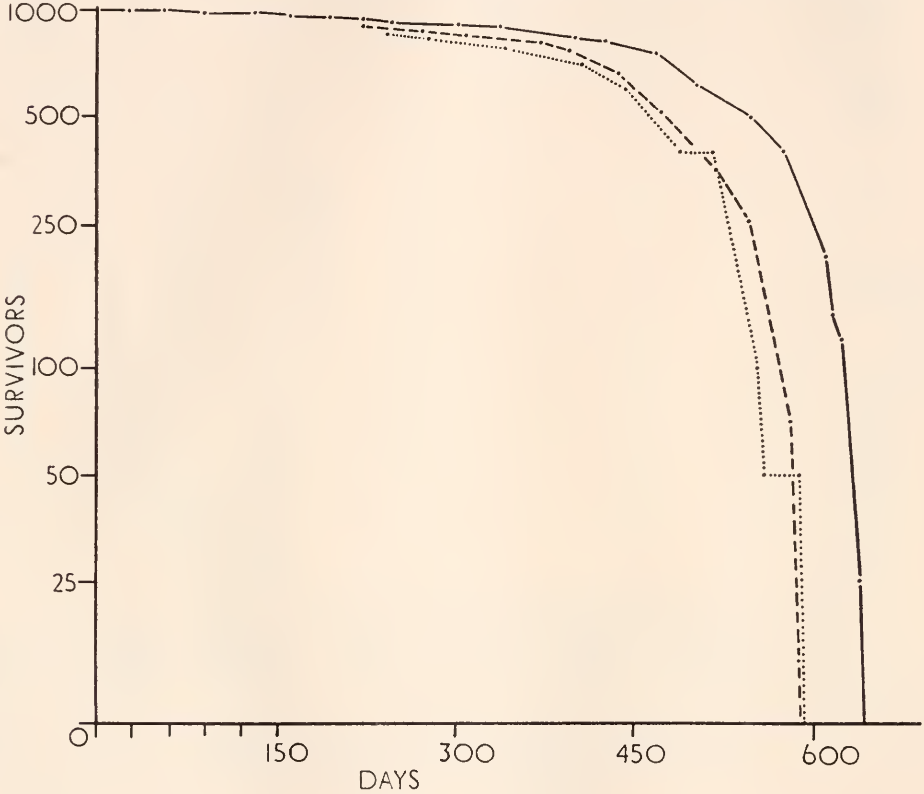

FIGURE 4. Corrected survival curves for three groups of ascidians which first appeared on the panels in August, September and October, 1957 (see Table III). ·————————· : Group A, appearing in August. ·- - - - - - - - - - - · : Group B, appearing in September. .... : Group C, appearing in October. For the sake of clarity curves B and C have not been carried back to the origin.

The curves shown in Figures 2 and 3 are complicated by a density-independent mortality due to fresh-water floods occurring between October 3 and 27, 1958. Following heavy rain a layer of low-salinity water penetrated the harbour and caused extensive mortality among sedentary organisms (Goodbody, 1961a). On panels at the 4-foot level 86% of the *A. nigra* were killed and at the 7-foot level 33% were killed. Subsequent to this mass mortality the mortality curve climbs steadily upward, but there is no reason to believe that this is not normal or that it is in any way connected with the floods. Data from 18 animals in a pilot experiment run in 1956–58 show that 50% had died in 12 months and all were dead after 85 weeks (19½ months).

TABLE III

Survival rate (1x) in three separate groups of A. nigra *showing the observed rate and the recalculated rate for a population free from density independent mortality (see text). Group A settled in August, 1957, B in September and C in October. Figures in parentheses show actual number of animals in each group at outset. For dates corresponding to period numbers see Table I. Periods added to this table are 18a: 6. 5. 59, 18b: 13. 5. 59, 18c: 20. 5. 59, 19a: 3. 6. 59*

Period No.	Observed Survival (1x)			Recalculated Survival (1x)		
	Group A (117)	Group B (210)	Group C (50)	Group A (117)	Group B (210)	Group C (50)
1.	1000	—	—	1000	—	—
2.	1000	1000	—	1000	1000	—
3.	1000	976	1000	1000	976	1000
4.	991	967	980	991	967	980
5.	991	957	980	991	957	980
6.	974	938	920	974	938	920
7.	974	919	920	974	919	920
8.	957	914	920	957	914	920
9.	940	900	920	940	900	920
10.	915	876	860	915	876	860
11.	897	848	840	897	848	840
12.	838	810	780	838	810	780
13.	350	262	300	811	769	755
14.	325	224	280	753	658	704
15.	265	176	240	614	517	603
16.	179	124	160	500	364	402
17.	145	90	160	405	264	402
18.	77	24	40	215	70	100
18a.	51	5	20	142	15	50
18b.	43	0	20	120	0	50
18c.	9	—	20	25	0	50
19.	9	—	20	25	0	50
19a.	0	—	20	0	—	50
20.	0	—	0	0	—	0

Since settlement of young animals goes on over a period of three months with small increments in later intervals, the population being dealt with cannot be considered as homogeneous in age structure. The information in Table I is therefore of limited interest only as a history of the entire population. It is more informative to consider separately the life table data for each of the three groups of animals settling in the first three months and to see if any differences exist between them. This is accomplished by a comparison of survivorship (1x) data and is shown in Table III and Figure 4 for the groups of animals which first appeared on panels in August, September and October, 1957.

Table III has been constructed so as to show the actual survival, and calculated survival data in which the effect of the density-independent mortality has been eliminated. To calculate the latter it has been assumed that the mortality index between October 3 and 27, 1958, would normally have been the mean of that prevailing in the periods immediately preceding and succeeding it; from this a value for the percentage mortality in period 13 has been calculated for each of the three groups. By substituting this value in place of the observed mortality in period 13 the lx values for periods 13–21 can then be re-calculated to give a picture of the survival curve for a population free from density-independent mortality.

Figure 4 shows corrected survival curves for the three populations. In constructing this figure it has been assumed that population A settled on August 1, B on August 28, and C on September 26, and the ordinate plots the number of days since settlement. In this way the three survival curves can be directly compared. This curve can be only an approximation to the true survival curve, but it is probably reasonably close. The obvious sources of error are in the calculation of the per cent mortality for period 13 and in assuming that all the animals in a group settled at the chosen dates. Furthermore, this does not include mortality in the first 28 days and is only a survival curve for animals of 28 days and over.

Two features of interest are apparent in Figure 4. First the survival curve in each case is of the "negative skew" rectangular type (Deevey, 1947) in which few deaths occur until very near the maximum length of life and most of the animals die in a short space of time near the end. This suggests senescence and physiological longevity and is discussed further below.

Secondly, although the form of the curve is similar in all three groups the first settlers appear to have lived for longer than the other two groups. The 50% level was reached after 550 days in Group A, 480 in Group B and 465 in Group C. There are three possible explanations for this difference. (1) The population might really be homogeneous, all having settled in the first week of August, and the early development of some of them may have been greatly retarded by, for example, food shortage, so that they appeared very much later than the others. This possibility, though real, can probably be ruled out. The maximum error that could have arisen this way is 58 days, while the difference in longevity is of the order of 85 days. (2) The first settlers may be genuinely more successful than later settlers and consequently live longer. (3) The sharp drop in the curve may be the result of a change in the environment and be ecological mortality and not senescence.

It is not possible at present to decide definitely between these two latter possibilities but in assessing them the following points may be considered. Life spans of 18–20 months have been calculated for several temperate water species of ascidians by Millar (1952, 1954) and Sabbadin (1957, 1958), and animals in a pilot experiment with *A. nigra* in 1956–58 had a total life span of 19–20 months. This supports the contention that the curve may be natural and senescent. On the other hand I have shown elsewhere (Goodbody, 1961c) that the climax sponge/anemone/ophiuran community inhibits the development of the early stages of the primary colonising community, possibly as a result of toxins produced by sponges or by competition for food. This "influence" might extend to the adult population of *A. nigra* and be responsible for its death. In support of this we may note that the final death of all three populations occurred at approximately the same time (Table III), and that this coincided with the time when the climax community was reaching full development. This problem can be resolved only by further experiment.

While there is a high rate of survival in populations settling in the first few months, the same does not appear to be true for animals which settle at later times. In Table IV the survival of animals to October 3, 1958 (*i.e.,* immediately before the floods) is shown for animals settling at different periods. There is a considerable and significant difference between those settling in August, September and October and those settling from November to June. Here is further evidence that *A. nigra* can survive only as a primary coloniser in the community, and that new individuals do not appear in the community because they cannot compete in it.

TABLE IV

Showing different rates of survival in groups of Ascidia nigra *settling at successively later stages. Survival has been taken to 3. 10. 58 (approximately one year) as density-independent mortality occurred on 6. 10. 58*

Settlement date	Number settling	Number surviving 3. 10. 58	% Surviving
Aug., 1957	117	98	84
Sept., 1957	210	170	81
Oct., 1957	50	39	78
Nov., 1957	11	5	45.5
Dec., 1957 to June, 1958	12	5	42

I know of no active predator on the adult ascidian and it appears that the test substance is distasteful. Fishes in an aquarium will eat the body tissue of an *Ascidia nigra* but examine and reject the test substance if that is fed to them. A small amphipod, *Erichthonius brasiliensis* (Dana) is common on the test and in and around the siphonal margin, but this is probably a true commensal arrangement. Several copepod commensals occur in the branchial sac and also a large pea-crab, *Pinnotheres moseri* Rathbun. The incidence of this crab in animals is very variable but there seems to be never more than one in any ascidian. There is no evidence that it harms the ascidian and all infected animals have appeared normal and healthy.

Externally a galatheid crab and the ophiuran, *Ophiothrix angulata,* are commonly found wandering on the test but have probably no effect other than to help keep the test clean. A large xanthid crab (*Menippe nodifrons* Stimpson) occasionally makes tunnels around the base of sessile organisms in this community and in a later experiment was suspected of actually dislodging some ascidians from a panel, but this is rare.

DISCUSSION

Previous studies of the life cycle of solitary ascidians have been published by Millar and Sabbadin, both of them concerned with temperate-water species. Millar (1952) studied *Ascidiella aspersa* and *Ciona intestinalis* in the Clyde, Scotland, and found the pattern of the life cycle of both species to be fairly similar. There is a single breeding season in summer, involving several generations of larvae; and the young animals grow until late autumn when growth ceases. In the following spring growth re-commences and these animals form the breeding stock for the subsequent summer spawnings. This adult population died off in the following winter, thus having a total life span of 12–18 months. In a later paper Millar (1954) studied populations of *Dendrodoa grossularia* from the Clyde and from Essex, England. This species has a similar form of life cycle, living for from 18 to 24 months, but in Essex has two distinct breeding seasons in each summer. Sabbadin (1957, 1958) studied *Ciona intestinalis, Molgula manhattensis* and *Styela plicata* in Venice, Italy. Breeding is continuous from early spring to late autumn in all three species. Animals metamorphosing in early spring may breed during the ensuing summer and have completed their growth within the year. Later settlers cease growing during winter, and do not complete their growth and breed until the following spring. The total life span is reported to be "about one year" (Sabbadin, *loc. cit.*).

Except for the similarity in total life span, the pattern of annual cycle in these temperate-water species differs from that of *Ascidia nigra*. Whereas the former have annual breeding seasons, *A. nigra* breeds throughout the year so that the population is composed of animals of all ages. Present information suggests that *A. nigra* commences breeding when it is about 85 days old and thereafter may spawn at intervals of about 60 days.

Growth in the temperate-water species is arrested during the winter months and according to Sabbadin (1957) is definitive. However, this author studied ascidian populations by means of monthly samples and his observations on growth are based entirely on these samples. From such data it is not possible to say with certainty that growth is not continuous throughout life. Unpublished data on *A. nigra* suggest that in Jamaica this species may continue to grow throughout life though at a progressively retarded rate. This point is important in relation to senescence and the problem of why ascidians die.

Life tables for other animals have been extensively reviewed by Deevey (1947) and by Allee *et al.* (1949). The point of interest here is in the type of survival that is illustrated. The curve shown in Figure 4 is typical of a population which exhibits senescence (Comfort, 1956) and very little ecological mortality. This raises again the question of why ascidians die. It has been mentioned earlier that the decline in the ascidian population is coincident with the rise of the climax sponge community. Experiments are now in progress to try and determine whether one is dependent on the other or whether the ascidians die for physiological rather than ecological reasons. In the studies by Millar and Sabbadin (*loc. cit.*) populations died over the winter months when water temperatures were cool. In these areas declining temperature may be the cause of, or hasten, the onset of death and thus obscure the incidence of senescence. In Jamaica, water temperatures vary about 6° C. throughout the year (Goodbody, 1961c) and are unlikely to be concerned in any way with death.

Finally it should be emphasized again that these data deal only with ascidians from the twenty-eighth day of life onwards and more drastic mortality is to be expected in the first four weeks. Data on this period are at present accumulating and will form the subject of a later paper in the series.

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SUMMARY

1. A "marked" population of *Ascidia nigra* has been followed throughout life from their first appearance to the time of death.

2. Mortality in the first four weeks after metamorphosis has not been studied. Thereafter few animals die until they are 18 months old and all are dead at 22 months old.

3. A mass mortality due to fresh-water floods occurred in the period of observation. A corrected survival curve has been calculated for a situation in which this did not occur. This suggests that senescence may occur.

4. *A. nigra* is a primary coloniser. When colonising new surfaces, the earliest colonisers survive longer than the later ones. Very few animals can colonise after the primary sessile community is three months old.

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FREEZING RESISTANCE IN SOME NORTHERN FISHES[1]

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Scholander *et al.* (1957) described two groups of marine fishes from Hebron Fjord, Labrador, which spend long periods at subfreezing temperatures. One group (deep-water fishes) appears to survive by remaining perpetually in the supercooled state. The second group (shallow-water fishes) is exposed to subfreezing temperatures for about two-thirds of each year and survives these periods by combining supercooling with some increase in the osmotic concentration of their body fluids. The former group lives in water depths such that ice is absent even during the coldest winters. The latter group, however, lives in shallower areas and is therefore likely to encounter ice. Natural selection has presumably operated on this second group so that those fishes have survived best which have added some antifreeze to their blood, thereby reducing the possibility of their fatally freezing as a result of an accidental collision with some ice. Several species of Norwegian boreal and arctic fishes apparently also respond to subfreezing temperatures in much the same way as the shallow water fishes from Labrador (Eliassen *et al.*, 1960).

The conditions of life faced by the shallow-water fishes in Labrador in winter give rise to several questions, such as: how can these fishes tolerate any degree of supercooling at all, living as they do in close proximity to ice (supercooled non-arctic fishes freeze rapidly when touched by a piece of ice (Scholander *et al.*, 1957)); and what is the nature of the antifreeze substance? It is not NaCl. The present paper describes new observations bearing on these subjects.

MATERIALS AND METHODS

In March, 1959, the present authors made a return visit to the Hebron Fjord. A small prefabricated laboratory hut was set up on the ice near Hebron settlement,

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and large numbers of fishes were caught by jigging with hand lines through holes in the ice over 2–10 meters of water. Water temperatures were between − 1.68° and − 1.81° C. Two species of fishes were taken: the short-horned sculpin (*Myoxocephalus scorpius*) and the Fjord cod (*Gadus ogac*). The sculpins weighed 200–800 gm.; the cod weighed 400–1000 gm.

Observations made on numbers of intact, living sculpins and cod were: body temperature, measured by thermometer inserted several centimeters into the cloaca; and survival time following contact with ice, either on the outer body surface, on the gills, or in the main body muscle mass by insertion of crystals under the skin.

Other fishes were immediately taken into the heated laboratory and blood samples taken by heart puncture. Heparin was added to a few samples, but most were simply allowed to clot in plastic centrifuge tubes, then stored on ice (for a maximum of three hours) until they could be centrifuged on an electric centrifuge. Following removal of aliquots for the analytical procedures carried out in the field, the plasma or serum samples were sealed into Pyrex glass ampules, frozen and returned in the frozen state to Gordon's laboratory. Frozen serum samples were obtained from 32 sculpins and 6 fjord cod.

Analyses made at Hebron were: freezing point depression, using the method described in Scholander *et al.* (1957); electrical conductivity, measured on 1 : 10 dilutions of serum with an Industrial Instruments, Inc., Model RC-16B conductivity bridge calibrated against known NaCl solutions; urea plus ammonia nitrogen, measured on two sculpin samples by the Conway microdiffusion technique; glucose, measured on two sculpin samples by the glucose oxidase method; glycerol, spot-tested on several sculpin samples using the acrolein reaction.

In April, 1960, Gordon visited the Biological Station of the Fisheries Research Board of Canada, St. Andrews, New Brunswick. Sea water salinities at St. Andrews were similar to those in the study area in Labrador. Water temperatures were 3–4° C. Winter water temperatures around St. Andrews rarely are as low as 0° C.

Eighteen short-horned sculpins were used to determine the tolerance of a non-arctic population of this species to water refrigerated to − 1.5° C. Four remaining sculpins were kept at 4° C. and serum samples were carried frozen to California.

Forty-two tomcod (*Microgadus tomcod*—a close relative of *Gadus ogac*) were used in several groups for low temperature tolerance experiments involving cooling from 4° to − 1.5° over periods of 12–48 hours. Blood samples were obtained from five tomcod which survived at − 1.5° for 9½ days. Sixteen other tomcod maintained at 4° C. were also used for blood samples. The serum obtained from all of these fish was also carried frozen to California.

The serum and plasma samples were used in attempts to identify the antifreeze substance. Samples were stored frozen at about − 20° C. and gradually used for chemical analyses over a two-year period. A series of general analyses (e.g., freezing-point depression, Na, K, Cl, non-protein nitrogen) were carried out on groups of samples from each species; then, as what appeared to be promising leads developed, more specific techniques were used. These specific techniques

TABLE 1

Analytical procedures used on fish plasma and serum samples

Analysis	Procedure
I. General analyses done on whole serum or plasma:	
Freezing-point depression[a,b,c]	Cryoscopy (Ramsay and Brown, 1955)
Chloride[a,b,c]	Volhard $AgNO_3$ — SCN titration
Sodium[a,b,c]	Flame photometer on diluted samples
Potassium[a,b,c]	Flame photometer on diluted samples
Total phosphorus[a,b]	Colorimetry (Feigl, 1947, p. 317; Chen *et al.*, 1956)
Urea[a,b]	Conway microdiffusion technique (Natelson, 1957, p. 387)
II. Analyses done on samples deproteinized with trichloracetic acid or (usually) ethanol: diethyl ether (3:1, V/V), some samples also desalted, either by electrodialysis or with pyridine:	
Non-protein nitrogen[a,b,c]	Nesslerization (Natelson, p. 272)
Amino nitrogen[a,b]	Colorimetry (Natelson, p. 93)
Glycerol[a]	Resorcinol test (Jones, 1947); paper chromatography (Block *et al.*, 1958, pp. 178, 182)
Ascorbic and dehydroascorbic acids[a]	Paper chromatography as for glycerol; colorimetry (Schaffert and Kingsley, 1955)
Reducing sugars[a]	Paper chromatography as for glycerol; Folin-Wu method (Natelson, p. 205)
Non-reducing sugars[a]	Paper chromatography (Block *et al.*, p. 185)
Aldehydes and ketones[a]	Paper chromatography (Block *et al.*, p. 340)
α-diketones[a]	o-dinitrobenzene test (Feigl, 1956, p. 24)
Carboxylic acids[a]	Paper chromatography (Block *et al.*, pp. 216, 231)
Amino acids[a,b,c]	Paper chromatography (Scherbaum *et al.*, 1959)
Aromatic compounds[a]	$AlCl_3$ test (Shriner and Fuson, 1948, p. 89)
Primary alkyl amines[b]	Rimini test (Cheronis and Entrikin, 1957, p. 260); 2, 4-dinitrochlorobenzene test (Smith and Jones, 1948, p. 110)
Secondary amines[b]	Nickel-dithiocarbamate test (Duke, 1945)
Tertiary amines[b]	N-bromosuccinimide test (Cheronis and Entrikin, p. 258)
Purines and pyrimidines[a]	Paper chromatography (Block *et al.*, p. 285); ultraviolet spectroscopy (see III below)
III. Analyses done on sculpin samples deproteinized as in II, vacuum-distilled to dryness at 30 ± 5° C., then redissolved in water or various organic solvents—in some cases two or three solvent extraction stages, with vacuum distillation to dryness between stages.	
pH[a]	pH meter on diluted samples
Ultraviolet absorption spectra[a,b,c]	UV spectrometer on pH 2, 7 and 11 water extracts dried and re-dissolved in ethanol:diethyl ether (3:1, v/v)
Infrared absorption spectra[a,b,c]	IR spectrometer, on extracts dissolved in absolute ethanol, $CHCl_3$, CCl_4, CS_2 and diethyl ether in various sequences
Simpler carboxylic acids and esters, including lipids[a]	Gas chromatography of methyl esters in ethanol, diethyl ether, $CHCl_3$ and CCl_4 extracts (courtesy J. Mead)
Amino acids[a]	As under III above, also by column chromatographic fractionation with identification on paper (courtesy K. Allen)

[a,b,c]: Species on which analyses done. [a] *M. scorpius*; [b] *G. ogac*; [c] *M. tomcod*

TABLE II

Plasma concentrations in the short-horned sculpin (Myoxocephalus scorpius)

Substance	Concentration [$\bar{x}$ ± S. E. (N)]		
	Labrador summer 4–7° C.	Labrador spring −1.7° C.	New Brunswick spring +4° C.†
Δ (mOsm/l.)	430 ± 10 (6)*	672 ± 15 (17) 775 ± 40 (6)	450 ± 5 (4)
Cl (meq./l.)	approx. 200*	234 ± 3 (6)	184 ± 2 (4)
Na (meq./l.)	—	216 ± 4 (6)	276 ± 2 (4)
K (meq./l.)	—	4.3 ± 1.7 (6)	6.4 ± 0.3 (4)
Total P (gm./l.)	—	0.55 ± 0.05 (5)	—
NPN (gm./l.)	—	1.3 ± 0.2 (5) 0.9 (1)*	1.7 ± 0.2 (4)
Urea-N (gm./l.)	—	0.4 ± 0.1 (3)	—
Amino-N (gm./l.)	—	0.15 ± 0.03 (4)	—

* Data from Scholander *et al.* (1957).

† Single pooled sample.

are summarized in Table I. This chemical identification effort was terminated with the exhaustion of the supply of samples.

RESULTS

Resistance to freezing in Labrador fishes

Data on osmotic concentration of the blood of fishes captured in Labrador in 1959 are included in Figure 1 and Tables II and III. These spring fishes were significantly more concentrated than the summer fishes studied by Scholander *et al.*

TABLE III

Plasma concentrations in codfish

Substance	Concentrations [$\bar{x}$ ± S. E. (N)]			
	Gadus ogac (Labrador)		*Microgadus tomcod*† (New Brunswick)	
	Summer, 4–7° C.	Spring, −1.7° C.	Spring, 4° C.	Spring, −1.5° C.
Δ (mOsm./l.)	430 ± 10 (6)*	505 ± 10 (5) 790 ± 10 (8)*	440 ± 10 (14)	525 ± 5 (5)
Cl (meq./l.)	approx. 200*	243 ± 19 (3)	142 ± 2 (14)	166 ± 3 (5)
Na (meq./l.)	—	216 ± 4 (3)	231 ± 3 (14)	246 ± 2 (5)
K (meq./l.)	—	5.5 ± 1.4 (3)	5.1 ± 1.4 (14)	8.3 ± 0.3 (5)
Total P (gm./l.)	—	0.72 ± 0.03 (2)	—	—
NPN (gm./l.)	—	4.0 ± 0.2 (3)	1.0 ± 0.2 (14)	1.3 ± 0.2 (5)
Urea-N (gm./l.)	—	0.7 ± 0.1 (1)	—	—
Amino-N (gm./l.)	—	0.25 ± 0.02 (3)	—	—

* Data from Scholander *et al.* (1957).

† Analyses on three pooled samples for 4° C. fish, one pooled sample for −1.5° fish.

The temperature of the water from which these fishes were taken varied from − 1.68 to −1.81° C. The body temperatures of fresh-caught fishes (measured within 30 seconds of their removal from the water) were uniformly − 1.50° C. for each of three sculpins, − 1.50 to − 1.75° C. for five cod.

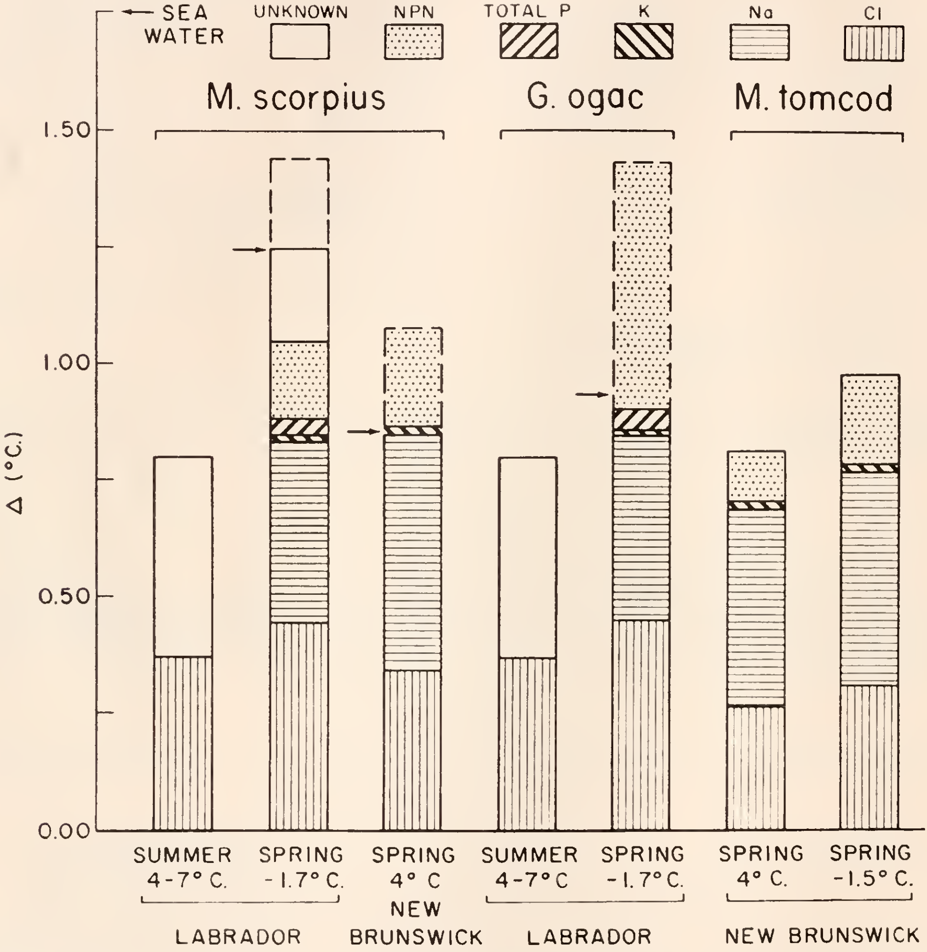

FIGURE 1. Blood serum concentrations of major constituents in groups of variously thermally acclimatized fishes of three northern species: *Myoxocephalus scorpius*, *Gadus ogac* and *Microgadus tomcod*. Measured concentrations converted to equivalent freezing point depression using 1 $M = 1.86°$ C. Total phosphorus (total P) and non-protein nitrogen (NPN) freezing point depressions calculated assuming one P or one N atom per molecule, respectively. Analyses for each component carried out on samples from 1–17 fishes in each group (*cf.* Tables II and III). Horizontal arrows alongside two *M. scorpius* bars and one *G. ogac* bar indicate measured freezing point depressions from 1959 (Labrador) and 1960 (New Brunswick) expeditions. Area above arrow outlined by dashes is, for spring Labrador *M. scorpius*, average measured freezing point depression from 1957 (Labrador) expedition. For spring New Brunswick *M. scorpius* and spring Labrador *G. ogac* similar areas above arrows indicate freezing point depression in excess of measured values calculated from chemical data.

It is evident that all of these fishes were supercooled by small but significant amounts—an average of 0.25° C. for the sculpins, about 0.75° C. for the cod. We therefore carried out experiments on the ease with which freezing could be induced in these fishes by seeding them with ice.

Three sculpins, damaged only very slightly by being jigged, were kept in the sea water in the fishing holes cut in the ice and observed for signs of freezing due to contact with the walls of the ice hole and with the ice particles carried over their gills by their respiratory movements. One sculpin died, showing ice crystals in one eye, within 30 minutes. The second sculpin died similarly after about one hour. The third sculpin showed ice crystals in one eye transitorily between 30 and 50 minutes after capture, then showed no further visible signs of freezing until its death after four hours. Death was unaccompanied by convulsions in these fishes.

Three cod similarly kept in an ice hole survived with no apparent freezing until observations ceased after 30 minutes. Five other cod handled similarly survived over an observation period of two hours. A sixth fish, seeded with snow and ice rubbed into a small incision in the skin of the back, died after about an hour with no visible signs of freezing. No convulsive movements were noted in any of these fishes.

Note should be made of the differences in mean osmotic concentration of the blood of the sculpins and cod taken in 1959 and those studied earlier by Scholander *et al.* We are sure that these differences are real, as the same procedures and some of the same people were involved in making both sets of analyses. The 1959 fishes were apparently significantly lower in internal osmotic concentration than were the fishes of the same species living in the same area two years earlier. Thus, while all shallow-water fishes seem to add some antifreeze to their blood during the winter, the amount added appears variable.

Resistance to freezing in New Brunswick fishes

Data on osmotic concentration of the blood of New Brunswick short-horned sculpins and tomcod are presented in Figure 1 and Tables I and II. The internal osmotic concentrations of these fishes were almost the same as the osmotic concentrations of Labrador sculpins and cod in summer, even though the New Brunswick fishes were sampled at the same time of year as were the springtime Labrador fishes. The addition of antifreeze to the blood of the sculpin, also probably the fjord cod, therefore appears to be a true response to low temperature and not simply a seasonal phenomenon. It is possible, however, that the New Brunswick fishes are physiologically different from the Labrador fishes.

New Brunswick fishes survived with difficulty when subjected to water temperatures as low as the environmental temperatures easily endured by Labrador fishes at the same time of year. At least the sculpins, however, probably can greatly improve their tolerance through gradual acclimatization.

Eighteen sculpins were transferred from sea water at 4° to sea water at − 1.5°, either directly or with some acclimation over periods longer than 12 hours. Seventeen of these froze upon coming in contact with bits of ice, whether this contact occurred immediately or after periods of up to 150 hours at − 1.50°. Only one fish, after 100 hours at − 1.5°, showed no signs of distress and did not freeze when vigorously rubbed with bits of ice at intervals over a period of an hour. This fish froze immediately, however, when cooled to − 1.7° in a bucket of sea water.

It therefore seems that only a small fraction of the New Brunswick sculpin population (one of 18 fishes tested) is able to develop resistance to freezing similar to that possessed by the entire Labrador fish population. More gradual acclimation might have increased this proportion somewhat, but it seems probable that New Brunswick sculpins are physiologically different from their arctic relatives on a population basis. Somewhat similar observations by Eliassen *et al.* (1960) on supercooled Norwegian *Cottus scorpius* (probably the same species as *M. scorpius*) indicate the existence of the same situation in non-arctic eastern Atlantic fishes as well.

Though closely related, the tomcod seems to be much less resistant to low temperatures and freezing than *Gadus ogac.* Only six fishes, of 42 cooled from 4° to − 1.5° over periods of 12–48 hours, survived unfrozen at temperatures lower than − 1.2°. One of these six survivors froze and died after 7 days at − 1.5°. The other five survived until the experiment was terminated with removal of blood samples after 9½ days. It seems probable, from the freezing point of the blood of these surviving fishes (Figure 1 and Table III), that the reason for their survival was chance avoidance of any contact with the few small bits of ice which formed in their tank.

The surviving tomcod at − 1.5° showed three differences from controls maintained at 4°. These were: (a) volume of blood obtainable by both cardiac and caudal artery puncture about four times larger in the warm- as opposed to the cold-acclimatized fish; (b) the blood of the cold-acclimatized fish appeared to have a higher hematocrit and clotted much more rapidly at room temperature than the blood of the warm-acclimatized fish; (c) the stomachs and intestines of only the five cold-acclimatized fish were swollen with what appeared to be sea water which the fish had drunk.

These differences between the high- and low-temperature groups of tomcod, combined with the higher blood concentrations of the low temperature fishes (Table III), make it reasonable to infer that the temperature of − 1.5° produced an osmoregulatory disturbance in the tomcod. This disturbance may have been due to an increase over normal levels of the permeability to water of the gill membranes or integument, or may have been a result of a slowing of rates of solute excretion.

Chemical nature of the antifreeze substances in Labrador fishes

The general comment summarizing the results of the chemical analytical work carried out is that we have not been able to specifically identify the antifreeze substance in either the sculpin or the cod. We have, along with Eliassen *et al.* (1960), verified the fact that the winter (low temperature) increase in blood concentration in both forms is not due to increased concentrations of NaCl. We have also eliminated from further consideration many classes of possible compounds and have some indications as to the directions in which work should go when additional material becomes available. The sum of blood Na, Cl, and K concentrations in New Brunswick sculpins at 4° was almost the same as the sum of these same concentrations in the blood of Labrador sculpins at − 1.7° (466 and 454 meq./l., respectively, Fig. 1 and Table II). The osmotic concentrations of these two groups of blood samples differed, however, by over 200 mOsm./l.

The picture in the fjord cod is not so clear, due to the lack of complete data from cod at higher temperatures. However, plasma Cl concentrations in winter cod were no more than 40 meq./l. higher than in summer fish. Osmotic concentrations differed by 75–360 mOsm./l.

An important biochemical difference between the short-horned sculpin and the fjord cod, a difference probably indicative of the nature of the antifreeze substance in the cod, is that shown by the non-protein nitrogen values for each form. Both species seem to have a great deal more NPN in their blood than do most other teleost fishes (NPN concentrations in the blood of many species of marine teleosts are in the range 0.04–0.73 gm./l. (Cordier and Chanel, 1958; Denis, 1922, Drilhon, 1952; Jonas and MacLeod, 1960), with only the Japanese eel in summer reaching a level as high as 1.25 gm./l. (Kawamoto, 1929)). In addition, however, the fjord cod seems to have about three times as much NPN as does the sculpin. Assuming all NPN substances in both species are in the form of molecules containing only one N atom, this fraction could supply more than enough solute to account for the wintertime increase in osmotic concentration in the fjord cod. It thus seems possible that the antifreeze of the fjord cod is a part of the NPN fraction. This is, however, not true for the sculpin, and NPN levels do not seem to vary significantly with temperature in this latter species.

Osmotically significant amounts of the compounds and groups of compounds listed in Table I were absent from the fjord cod blood samples tested. All amine tests were negative, even when samples had been treated with powdered zinc in order to reduce any oxides which may have been present (*e.g.*, trimethylamine oxide).

A point of interest concerning the fjord cod samples is the identity of the commonest free amino acids which were present. These were: aspartic and glutamic acids, threonine and monoiodotyrosine. Small quantities of samples were used in all of these analyses, so it is probable that acids present in very low concentrations were missed.

The identity of the sculpin antifreeze is presently completely obscure. There is a distinct possibility that no one compound is the antifreeze. If it is a single substance, it is apparently not a part of the NPN fraction and is probably not a phosphorus-containing compound. Other compounds and groups of compounds tested for but not detected in osmotically significant amounts are listed in Table I. Analysis of the amino acids present in sculpin blood showed alanine, methionine and taurine to be present in largest quantities.

Chemical responses to low temperatures in New Brunswick tomcod

It is interesting to note that plasma NPN concentrations in tomcod maintained at − 1.5° apparently increased by a proportionately larger amount than did the freezing point depression of the blood or the concentrations of the commonest inorganic ions. Plasma NPN levels rose about 30% while osmotic concentration increased only about 20%, chloride increased about 15% and sodium increased about 6% (Fig. 1 and Table III). The compounds involved in this increase in concentration are unidentified. There is a striking similarity between the tomcod's response to low temperature and that shown by the fjord cod.

DISCUSSION

The degree of supercooling occurring in all arctic fishes studied to date is quite small. However, even this slight degree of supercooling carries with it significant danger of fatal freezing if seeding with ice should occur. In order for the body fluids of the Labrador sculpins to come to thermodynamic equilibrium with the fishes' own body temperatures, approximately 20% of their free body water would have to freeze. For the fjord cod the equivalent figure would be about 40–50% of free body water.

We have no way of estimating how much, if any, of the body water actually did freeze in the Labrador sculpins and fjord cod tested for survival in the presence of ice. All we know is that these fishes did survive for periods of hours in contact with ice. There were none of the usual visible signs of freezing which always occur almost immediately in non-arctic fishes subjected to similar treatment (*cf.* Scholander *et al.*, 1957). It therefore seems probable that if there was any freezing of body fluids, it occurred slowly and probably did not spread very far through the tissues of the fishes' bodies. A similar situation apparently existed in some of the fishes studied by Eliassen *et al.* (1960).

Several theoretical alternatives seem reasonable as possible explanations for these observations. First, one might postulate that the skin, gills, etc., of the Labrador sculpins and cod are less open to penetration by ice crystals than the integuments of non-arctic fishes were shown to be by Scholander *et al.* (1957).

Second, it is possible that the skins of arctic fishes are no less penetrable by ice than are those of non-arctic forms, but that their body fluids possess special properties. One such property might be a significant slowing of ice crystal growth. The antifreeze substances themselves might confer this property, as do glycerol and other alcohols and various sugars, also some proteins in pure solutions (Lusena, 1955). Another special property might be the occurrence of larger amounts of bound water around tissue proteins, etc., than are present in non-arctic forms.

A third possibility is that neither of the above suggestions is correct, but that instead the spread of ice crystals through the bodies of our fishes was inhibited by the cell membranes of the fishes' tissues, and no damaging intracellular freezing occurred. The cell contents in this circumstance would have to tolerate some dehydration. Possible support for this idea comes from the observations of Chambers and Hale (1932) on the efficiency of frog sarcolemmae and amoeba cell membranes as barriers to propagation of ice crystals.

In both of the last two situations it would seem possible for fishes which had been seeded by a chance encounter with ice, as perhaps in an effort to escape a pursuing predator, to rid themselves of such ice as may form internally by becoming physically active enough temporarily to raise their body temperatures by a few tenths of a degree. The body temperatures we measured on the Labrador sculpins indicate that they may generally be a few tenths of a degree warmer than their environment. Observations by Britton (1924) indicate that similar small differences between body and ambient temperatures may be a year-round feature of *M. scorpius* even in non-arctic areas.

SUMMARY

1. The occurrence of small degrees of supercooling and the presence in the blood of organic antifreeze compounds are confirmed in arctic populations of short-horned sculpins (*Myoxocephalus scorpius*) and fjord cod (*Gadus ogac*) captured at Hebron Fjord, Labrador, in early spring. The quantity of antifreeze added seems variable, however.

2. Although significantly supercooled, these same fishes were found to be very resistant to freezing even though seeded with ice. Possible explanations for this resistance are discussed.

3. Non-arctic populations of the sculpin and of the tomcod (*Microgadus tomcod,* a close relative of the fjord cod) also studied in early spring were found to lack both the resistance to freezing when supercooled and also the antifreeze substances found in the arctic fishes. Very few of the non-arctic sculpins were able to develop any resistance to freezing even after several days' exposure to arctic water temperatures.

4. The antifreeze substance added to the blood of the fjord cod is indicated to be a member of the non-protein nitrogen fraction. There is no evidence that it is an amino acid, an amine or an amine oxide.

5. The antifreeze substance added to the blood of the short-horned sculpin is also unidentified. It apparently contains neither nitrogen nor phosphorus, is not glycerol or a related alcohol and probably is not a reducing or a non-reducing sugar, an aldehyde or ketone, a carboxylic acid (lipid or other), an ester or an aromatic compound.

6. Tomcod exposed to arctic water temperatures show increases in plasma non-protein nitrogen levels similar to those which occur in the fjord cod in winter.

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A HISTOCHEMICAL STUDY OF DIGESTION AND DIGESTIVE ENZYMES IN THE RHYNCHOCOELAN LINEUS RUBER (O. F. MÜLLER)

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It has been shown in a previous account (Jennings, 1960) that digestion in the rhynchocoelan *Lineus ruber* is the result of both extracellular and intracellular processes. The food, which consists of animals such as small annelids and crustaceans captured by means of the eversible proboscis, is swallowed whole and, after being killed by acid secretions poured on to it during its passage through the foregut, is broken down into a semi-fluid mass within minutes of arrival in the intestine. The enzyme bringing about this initial, extracellular, breakdown is produced by gland cells scattered throughout the intestinal gastrodermis and operates at a pH of 5.0–5.5. The fragmenting food is phagocytosed by other gastrodermal cells and digestion completed intracellularly.

In the present work the course of digestion has been examined in greater detail and an attempt made to identify some of the enzymes concerned in both the breakdown of the food and the general metabolic activity of the gastrodermis.

MATERIALS AND METHODS

Individual *Lineus ruber* were isolated and starved for seven days to clear the gut of all traces of previous meals. Individual isolation was necessary since after five or six days without food cannibalism often occurs.

To study the course of digestion, and to locate and identify the enzymes concerned, starved *Lineus* were fed upon inert test foods such as clotted frog blood, either alone or mixed with cooked beef fat or starch paste. These foods were used in preference to the natural living food to eliminate any possibility of enzymes contained in the latter being mistaken for those produced by the *Lineus* gut.

Series of *Lineus* were killed for examination after seven days' starvation and at progressive intervals up to 48 hours after an observed meal on one or other of the test foods. In earlier experiments the *Lineus* were killed by freezing in isopentane cooled by liquid nitrogen to $-160°$ C. and subsequently dehydrated for 48 hours at $-40°$ C. under a vacuum of 10^{-3} mm. mercury. Such specimens were embedded in paraffin wax (melting point 42° C.), sectioned at 8 μ and examined by the histochemical techniques listed below. During the course of the work, however, it was found that specimens fixed for 12 hours at 4° C. in 10% formalin in sea water, buffered to pH 7.0, and then rapidly dehydrated in absolute acetone at the same temperature, cleared in xylol at room temperature and embedded in 42° C. wax showed no significant decrease in enzyme activity when compared with the freeze dried specimens. The duration of dehydration, clearing and embedding was kept to the absolute minimum consistent with the size of the specimen. This method of preparing sections for histochemical examination was adopted for the bulk of the work as it enabled many more specimens to be dealt with in a given time and was far less troublesome in operation.

The sections were mounted with albumen and after drying at 20° C. for six hours were dewaxed in xylol and passed through two changes of absolute acetone before being transferred directly into the various reagents for visualising enzyme activity.

Carbonic anhydrase activity, known to be associated with production of hydrochloric acid in the mammal stomach, was visualised by the cobalt sulphate-bicarbonate method given by Hausler (1958). Sections were incubated for three hours at 20° C. and it was found that for optimum results the layer of substrate solution upon the sections must not exceed 1 mm. in depth. The medium was buffered to pH 8.0 and control sections incubated in the presence of 4×10^{-3} *M* Diamox sodium (2 acetylamino-1,3,4-thiadiazole-5-sulfonamide sodium), a specific inhibitor for carbonic anhydrase. As a further control, and to check the reliability of the method, sections of mouse stomach were similarly treated.

Proteolytic enzymes were investigated by the methods of Hess and Pearse (1958) for cathepsin C type enzymes and Burstone and Folk (1956) for leucine aminopeptidase. To detect cathepsin C type activity, sections were transferred from acetone into a 10^{-5} *M* solution of E-600 (diethyl-p-nitrophenyl phosphate) to inactivate esterases whose presence would otherwise give false positive reactions. The sections were then incubated for three to four hours at 20° C. in a standard indoxyl acetate medium buffered at pH 5.0. Control sections were immersed for one hour, before incubation, in 1×10^{-3} *M* cysteine solution which activates cathepsin C so that sections treated in this way show a more intense enzymatic action than do non-treated ones. Further controls were performed by immersing sections in 1×10^{-3} *M* lead nitrate solution for one hour, or in water at 90° C. for three minutes, before incubation. For leucine aminopeptidase activity sections were incubated for six hours at 20° C. in a medium containing L-leucyl-β-naphthylamide as substrate and Garnet G.B.C. as a simultaneous coupler. The medium was buffered to pH 7.2 and heat-inactivated sections used as controls.

Lipolytic activity was investigated by the method of Gomori (1952) using Tween 80 as the substrate in a medium buffered to pH 7.2. Sections of *Lineus* fed on blood mixed with beef fat were incubated for twelve hours at 20° C. and again heat-inactivated sections were used as controls.

Attempts were made to detect carbohydrase activity by using the ferric-8-hydroxyquinoline method for β-glucuronidase as modified by Billett and McGee-Russell (1955). This method failed to give satisfactory results and observations on carbohydrate digestion were limited to tracing the fate of starch meals by the Lugol's iodine technique.

Alkaline phosphatase activity was demonstrated by the calcium phosphate method (Gomori, 1952). Sections were incubated for two hours at 20° C. in a medium containing sodium β-glycerophosphate buffered to pH 8.0 and control sections inactivated by heat prior to incubation. Sections were also examined for acid phosphatase activity (Gomori, 1952), again using sodium β-glycerophosphate as substrate.

OBSERVATIONS

Histology of the gut

The histological structure of the gut in *Lineus* has been described in detail elsewhere (Jennings, 1960). Briefly, the gut consists of three regions, the mouth and buccal cavity, the foregut, and the intestine. It is ciliated throughout its length and lacks both multicellular glands and musculature. The buccal cavity is lined by ciliated cuboidal cells backed by masses of acidophil and basophil gland cells, the majority of which are Alcian blue- and periodic acid-Schiff-positive and produce mucus to facilitate ingestion. The foregut is lined by similar ciliated cells, interspersed with acidophil gland cells, lying upon tissue with indistinct cell walls containing numerous acidophil and basophil glands, many of which are mucus-producing. The intestine forms the major portion of the gut and bears paired serially repeated lateral pouches throughout its length. The intestinal wall, or gastrodermis, is made up of two types of cells which stand in a single layer upon a thin basement membrane. The larger and more numerous cells are columnar and the cytoplasm usually contains phagocytosed food particles in various stages of digestion. The second type of cell is glandular and contains up to 30 acidophil proteinaceous spheres which are discharged into the gut lumen when food enters.

Enzymes produced in the gut

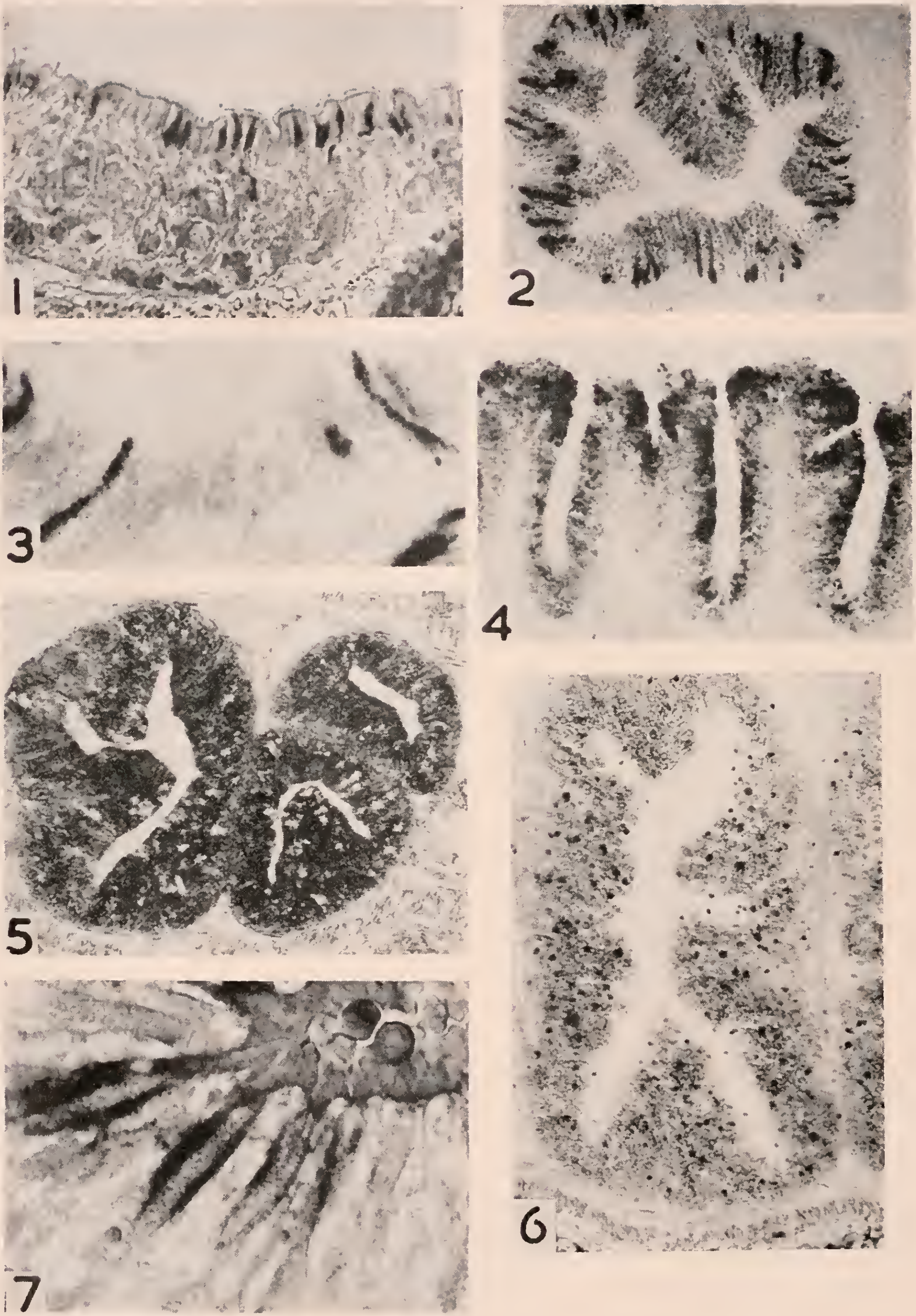
Carbonic anhydrase

Carbonic anhydrase activity was found in some 10–15% of the acidophil gland cells of the buccal cavity and foregut (Fig. 1). In the latter, gland cells in both the lining epithelium and the backing syncytial tissue gave positive reactions. The number of reactive cells, and the degree of activity, could not be related to the time of feeding and it would appear that the enzyme is always present in an active condition. Control sections incubated in the presence of the specific inhibitor, Diamox sodium, gave no reaction. Sections of mouse stomach incubated at 37° C. showed intense activity in the acidophil oxyntic cells of the fundic glands, which are known to be the source of the gastric hydrochloric acid. This activity, as in *Lineus*, was inhibited by Diamox sodium.

Cathepsin C

During starvation the gland cells of the gastrodermis give an intense positive reaction to the Hess and Pearse method for cathepsin C (Figs. 2 and 3). The individual spheres within the gland cells stained so intensely that often other details of the cell, such as the nucleus, were obscured. Sections immersed in 10^{-3} M cysteine solution, a known activator of cathepsin C type enzymes, before incubation, reached the maximum density of staining in approximately half the incubation time needed by non-activated sections. Sections inactivated by heat or by immersion in 10^{-3} M lead nitrate solution gave no reaction whatsoever.

Sections prepared within 30 minutes of feeding showed that the majority of the gland cells had discharged their spheres and such cells failed to give any reaction. This condition (Fig. 4) persisted for two to three hours and then the



Figs. 1–7.

gland cells gradually filled up again with active spheres so that about six hours after feeding they were back in their normal condition. After a meal of frog blood haemolysis occurred as the food entered the intestine, and within 30 minutes the digesting mass in the lumen gave a fairly strong reaction for cathepsin C. About this time the columnar cells of the gastrodermis commenced phagocytosis of the food and the phagocytosed material continued to give a cathepsin reaction within the cells (Fig. 4). This, however, decreased rapidly with time and it was clear that the reaction was the result of enzyme being phagocytosed with the food and remaining active for a short time within the cells. There was no evidence of intracellular production of cathepsin C. One and a half hours after feeding, the gastrodermis was loaded with phagocytosed material and the apparently intracellular cathepsin activity had disappeared.

The optimum pH for visualisation of the enzyme was 5.0 and this agrees with previous *in vivo* observations using indicator-stained food, which showed that the early stages of digestion went on at pH 5.0–5.5 (Jennings, 1960).

Leucine aminopeptidase

Sections of starved *Lineus*, and of specimens fixed within one and a half hours of feeding, gave no reaction for leucine aminopeptidase. About two hours after feeding, however, phagocytosed material started to show a faint positive reaction. The intensity of the reaction increased rapidly with time, and six hours after feeding all the material in the columnar cells of the gastrodermis gave an extremely strong and vivid reaction (Fig. 5). Heat-inactivated control sections showed no such activity. At no time was any leucine aminopeptidase activity found in either

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FIGURE 1. Longitudinal section of the foregut in *Lineus*, showing carbonic anhydrase activity in the gland cells of the ciliated epithelium. Hausler cobalt sulphate-bicarbonate method. Scale: 1 cm. = 50 μ.

FIGURE 2. Transverse section of an intestinal pouch in a starved *Lineus*, showing the positive cathepsin C type reaction given by the gland cells of the gastrodermis. The gland cells are seen as dark streaks. Hess and Pearse E600-indoxyl acetate method. Scale: 1 cm. = 50 μ.

FIGURE 3. Transverse section of a portion of the gastrodermis of a starved *Lineus*, showing two gland cells packed with enzymatic spheres giving an intense cathepsin C type reaction. Hess and Pearse method. Scale: 1 cm. = 25 μ.

FIGURE 4. Longitudinal section of the gastrodermis in *Lineus* 30 minutes after feeding. Hess and Pearse method. The gland cells have discharged their spheres and are no longer apparent by this technique. The columnar cells have commenced phagocytosis, and the newly engulfed food, seen in the distal regions of the cells, shows cathepsin C type activity retained from the initial extracellular phase of digestion. Scale: 1 cm. = 50 μ.

FIGURE 5. Transverse section of two intestinal pouches in *Lineus* six hours after feeding. The gastrodermis is swollen and loaded with food vacuoles all exhibiting intense leucine aminopeptidase activity. Burstone and Folk L-leucyl-β-naphthylamide G.B.C. method. Scale: 1 cm. = 50 μ.

FIGURE 6. Transverse section of an intestinal pouch in *Lineus* four hours after a meal of frog blood and beef fat. The gastrodermis is loaded with food vacuoles, many of which show lipolytic activity (seen as black spheres). Gomori Tween 80-lead sulphide method. Scale: 1 cm. = 50 μ.

FIGURE 7. Transverse section of a portion of the *Lineus* gastrodermis within 5 minutes of feeding, showing intense alkaline phosphatase activity around the gland cells. The gut lumen (top right) contains haemolysing frog erythrocytes. Gomori calcium phosphate method. Scale: 1 cm. = 25 μ.

the gland cells of the gastrodermis or the gut lumen, and this enzyme would appear to be entirely intracellular and to be concerned only in the later stages of digestion. The optimum pH for the visualisation was *7.2* and this difference in pH optima between the lumen-acting cathepsin C and the intracellular aminopeptidase is probably the reason why aminopeptidase activity does not commence immediately phagocytosed material enters the cells. It has been seen that newly phagocytosed food continues to show cathepsin C activity for over one hour after feeding and so is presumably still at, or near, the acidic pH value necessary for this. The decrease of cathepsin C activity and its gradual replacement by aminopeptidase will be accompanied by an increase in pH value up to the optimum *7.2* and this apparently takes a little time to achieve.

Leucine aminopeptidase activity continues whilst food remains in the gastrodermis and finally disappears 9 to 12 hours after feeding, depending upon the amount of food taken. It would appear, therefore, that this enzyme is normally present in an inactive form, unlike the cathepsin C of the gland cells, and only becomes active (*i.e.*, as shown by the present method) when it is secreted from the cytoplasm into a food vacuole.

Lipase

Small amounts of lipolytic activity were found in the gastrodermis and parenchyma during all stages of starvation. These were probably the result of the starved *Lineus* utilising its fat reserves which are laid down in these sites (Jennings, 1960).

No significant increase in activity occurred until two to three hours after a meal of blood and beef fat. About this time a few food vacuoles resulting from phagocytosis of the meal showed a positive reaction. The number of such vacuoles then increased rapidly and within a further hour they could be found throughout the gastrodermis (Fig. 6). As intracellular digestion progressed the number of reactive vacuoles diminished but some could be found up to 48 hours after feeding. This was a consequence, no doubt, of the unusually large proportion of fat in the meal.

The optimum pH for the reaction was *7.2–7.4* and again, as in the case of aminopeptidase, this is probably the reason for the time lag between the onset of phagocytosis and the appearance of lipolytic activity.

Control sections inactivated by heat showed no lipolysis.

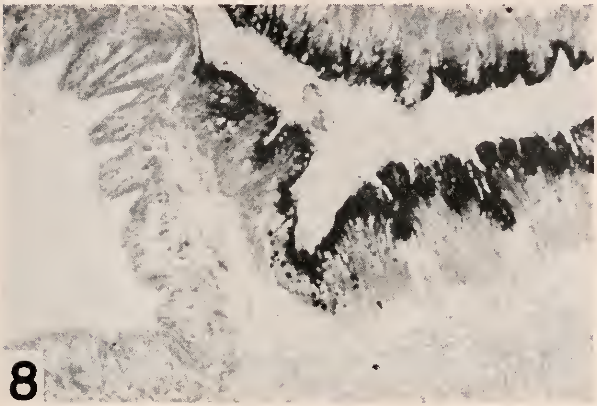
Carbohydrase activity

Attempts to localise β-glucuronidase activity by the Billett and McGee-Russell method during starvation and after meals of blood and starch paste were unsuccessful. The end product of the histochemical reaction, ferric-8-hydroxyquinoline, was precipitated indiscriminately over the entire sections, both experimental and control. Sections stained with Lugol's iodine, however, showed progressive digestion and disappearance of phagocytosed starch parallel in time with the aminopeptidase and lipase activity, and it was concluded that the unknown carbohydrases act at a similar slightly alkaline pH. There was no extracellular carbohydrate digestion.

Alkaline phosphatase

The gland cells of the gastrodermis normally show no alkaline phosphatase activity but immediately after the *Lineus* has fed, when they are discharging their enzymatic spheres into the gut lumen, intense alkaline phosphatase activity appears around the cell walls (Fig. 7). This localised activity disappears during reconstitution of the gland cells and so appears to be associated with their secretory rather than recovery phase.

The columnar cells of the gastrodermis show at all times intense alkaline phosphatase activity along their free ciliated borders (Fig. 8). The zone of activity varies in depth from a narrow band 3–5 μ deep immediately below the cilia, to a broad belt which may extend down into the cells for as much as one-third of their depth. This activity is obviously concerned with some aspect of the digestive function of the gastrodermal cells, for it is completely absent from the cells of the foregut wall (Fig. 8).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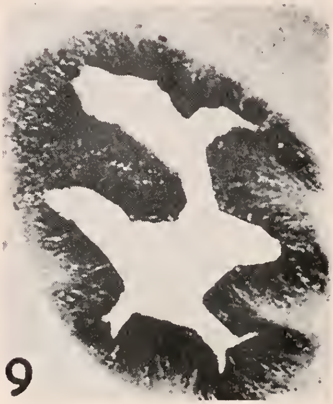
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FIGURE 8. Longitudinal section of a portion of the foregut (left) and intestine (right) of a starved *Lineus*. Gomori method for alkaline phosphatase. The cells of the foregut show no activity, in marked contrast to those of the intestinal gastrodermis which show intense activity distally. Scale: 1 cm = 50 μ.

FIGURE 9. Transverse section of an intestinal pouch of *Lineus* four hours after feeding. All the food vacuoles and some of the cytoplasm surrounding them show intense alkaline phosphatase activity. Gomori method. Scale: 1 cm. = 50 μ.

The distal band of alkaline phosphatase activity persists during extracellular digestion but as phagocytosis and intracellular digestion advance, it spreads downwards until the entire cytoplasm of the columnar cells gives a positive reaction. The activity is more concentrated around and within the food vacuoles (Fig. 9) and reaches its peak at about the same time as aminopeptidase and lipase activity. Thus it would appear to be connected with the secretion of these enzymes and the absorption of the products of intracellular digestion. This condition persists until digestion is completed and then the activity decreases in amount until only the normal distal zone remains.

Acid phosphatase

No trace of acid phosphatase activity was found in any region of the gut

DISCUSSION

Whilst it is clear that many more enzymes than the ones located in this work will be concerned in digestion in *Lineus,* sufficient information has been obtained for the sequence of digestive processes and the part played by the different types of enzymes to be understood.

The presence of carbonic anhydrase in acidophil gland cells of the buccal cavity and foregut would suggest that these cells are the source of the acid secretions poured on to the food during ingestion to assist in killing it and to provide a medium of suitably low pH value for the initial stages of digestion, taking into account the known association of this enzyme with hydrochloric acid secretion in the oxyntic cells of the mammal. The inhibition of the enzyme by the specific inhibitor for carbonic anhydrase and the similar results obtained with mouse oxyntic cells leave little doubt as to its identity. It was suggested previously that certain basophils in the foregut wall produced the acid secretions (Jennings, 1960) but since these fail to show carbonic anhydrase activity it may be that they have some other, unknown, function.

The early, extracellular, stages of digestion are effected by the proteolytic enzyme discharged from the gland cells of the gastrodermis. This has been identified as cathepsin C, or, at least, a cathepsin C *type* enzyme, and the identification confirmed by use of specific activators and inhibitors. Cathepsin C is an endopeptidase and attacks inner portions of protein chains to break down the molecule into simpler polypeptides and peptides. Thus its function in *Lineus* is to initiate proteolysis and break down the food to a condition suitable for entry into the gut cells where digestion is completed. In this respect the enzyme has an adaptive significance comparable to that of the elaboration of the feeding mechanism in the triclad Turbellaria, where purely mechanical means are used to make the food available for phagocytosis, and extracellular digestion does not occur (Jennings, 1957). In *Lineus*, and presumably most rhynchocoelans, a simpler type of feeding mechanism means that the food is swallowed whole and consequently there must be some other provision for its breakdown before intracellular digestion can begin.

The intracellular proteolysis appears to be effected by aminopeptidases, of which one example, leucine aminopeptidase, has been identified. These enzymes are exopeptidases and remove terminal amino acids from polypeptides resulting from endopeptidase activity and so complete digestion of the protein content of the food. They function at a slightly alkaline pH, in contrast to the extracellularly-acting endopeptidase, cathepsin C, which requires a fairly strongly acidic medium. Thus proteolysis in *Lineus* resembles that in most other animals in that it occurs in two distinct phases, the first acidic, the second alkaline.

The extracellular and intracellular proteolysis makes the fat and carbohydrate content of the food available for digestion by breaking down tissue and cell membranes, and the digestion of these food elements is entirely intracellular. Lipolytic activity appears at about the same time as aminopeptidase and the enzyme responsible operates at a similarly slightly alkaline pH. It is probable that the enzyme visualized here is the "true lipase" of Gomori (1952), homologous with mammalian pancreatic lipase, since an unsaturated substrate (Tween 80) was used and this, according to Gomori, is attacked only by pancreatic type lipase and not by other esterases.

It was not possible to identify any carbohydrases but the intracellular digestion of starch showed that these are present and working at a pH similar to that needed for aminopeptidase and lipase activity. Rosenbaum and Rolon (1960) located β-glucuronidase activity in the phagocytic gut cells of the not too distantly related triclad flatworm and it may be that this enzyme is also present in *Lineus* but failed to survive fixation.

The intense alkaline phosphatase activity observed in the distal region of the columnar gastrodermal cells appears to be related to the part played by the cell wall and its cilia in the phagocytic uptake of food. The cilia coalesce into pseudopodia-like processes which engulf the fragmenting food (Jennings, 1960) and the alkaline phosphatase no doubt plays some part in this modification and later recovery of the cilia. This interpretation is supported by the absence of phosphatase activity from the foregut cells, which are not concerned in uptake of food and whose cilia show no such modification. The alkaline phosphatase activity developing deeper within the gastrodermal cells during intracellular digestion would seem to be related to secretion from the cytoplasm into the food vacuoles of aminopeptidases, lipase and carbohydrases since these appear simultaneously with the phosphatase.

SUMMARY

1. Digestion in the rhynchocoelan *Lineus ruber* is both extracellular and intracellular. The extracellular phase is entirely proteolytic and is brought about by an endopeptidase acting in an acid medium. The semi-digested food is then phagocytosed and digestion is completed in the second, intracellular phase by exopeptidases, lipases and presumed carbohydrases, all operating in an alkaline medium.

2. The following enzymes have been located and identified by histochemical methods: carbonic anhydrase, a cathepsin C type protease (endopeptidase), leucine aminopeptidase (exopeptidase), lipase and alkaline phosphatase.

3. Carbonic anhydrase occurs in acidophil gland cells in the buccal cavity and foregut. It is believed to be associated with production of acid used to kill the food and provide a suitable medium for the extracellular phase of digestion.

4. The cathepsin C type protease is produced by gland cells in the gastrodermis and is discharged into the gut lumen to bring about the initial extracellular proteolysis.

5. Leucine aminopeptidase is produced within the phagocytic cells of the gastrodermis when food vacuoles are present and is concerned in completion of protein digestion.

6. Lipase, identified as "true lipase" homologous with mammalian pancreatic lipase, is formed within the phagocytic cells at the same time as leucine aminopeptidase and attacks the fat content of the food.

7. Alkaline phosphatase is present in an active form in the gastrodermis and appears to be concerned with phagocytosis. The amount present increases when intracellular digestion occurs and this increase is apparently associated with secretion of aminopeptidase and lipase into the food vacuoles.

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NEUROSECRETION AND CRUSTACEAN RETINAL PIGMENT HORMONE: DISTRIBUTION OF THE LIGHT-ADAPTING HORMONE[1]

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Pigmentary effectors of crustaceans, the chromatophores and retinal pigments, have long been known to be regulated by hormonal substances originating in the eyestalks (Perkins, 1928; Kleinholz, 1936; Fingerman *et al.*, 1959). Localization of the origin of these substances within the eyestalks is less well known. Hanström (1937) put these physiological observations on a firmer morphological basis by describing two structures in the crustacean eyestalk that might be involved in regulating the integumentary chromatophores: (1) the X-organ, composed of modified sensory cells of a rudimentary eye papilla, and (2) the sinus gland, usually located dorsally between the two middle optic ganglia. After testing extracts prepared from portions of eyestalks containing one or the other of these two glandlike structures, Hanström (1937) and Carlson (1936) proposed the sinus gland rather than the X-organ as the source of chromatophorotropic activity. Initial observations by Brown (1940) comparing activities of sinus gland extracts, of extracts prepared from entire eyestalks, and of extracts prepared from eyestalks from which sinus glands had been removed, indicated that nearly all the chromatophorotropic activity could be accounted for by the sinus gland. Later, however, more quantitative studies showed that extracts prepared from sinus-glandless eyestalks or from the four optic ganglia were as active on crustacean chromatophores as those prepared from sinus glands alone (Brown, 1950; Sandeen, 1950).

Little is known about the localization of the light-adapting retinal pigment hormone. Welsh (1941) found extracts of the sinus gland and of the medulla terminalis to be active, while those of supraesophageal ganglia were not; the activity of medulla terminalis extracts was explained as possibly due to residual tissue from the sinus gland, or to material that had escaped from the sinus gland during its removal from the eyestalk tissue. Kleinholz (1958), however, found little or no retinal pigment activity in sinus gland extracts in some species, while substantial amounts of this hormone were found in components of the eyestalk other than the sinus gland, and in other ganglia of the central nervous system.

Cytological examination of the sinus gland had disclosed a surprising lack of cellular organization in what was supposedly an active endocrine gland. Largely as a result of the studies of Passano (1953) and of Bliss and Welsh (1952) a revised explanation of the morphological relationships of the sinus gland was pro-

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posed, based on the description of a new X-organ (Carlisle and Passano, 1953), differing in its histology and in its location in the eyestalk from the one originally described by Hanström. This new X-organ, located in the fourth optic ganglion, is composed of modified neurons, the axons of which constitute the so-called sinus gland nerve (Bliss and Welsh, 1952; Passano, 1953). Passano has interpreted the sinus gland as consisting of an aggregation of these axonal terminals, swollen by the accumulation of neurosecretory materials originally elaborated by such modified neurons and transported to their final site by axoplasmic flow. Bliss, Durand and Welsh (1954) have reported that axons from neurosecretory cells at other locations in the central nervous system also contribute to the formation of the sinus gland.

In view of the variety of hormonal functions now attributed to this sinus gland-X-organ complex, and the inadequacy of information on the origin of such hormones, it was believed desirable to undertake such localization studies with the hope that correlation of particular hormones with specific cell types in this anatomical complex might subsequently be possible.

Materials and Methods

A variety of decapod crustaceans was used in this study. Donor animals whose eyestalks and eyestalk components were tested for distal retinal pigment hormone included the following: *Libinia emarginata* Leach, *Callinectes sapidus* Rathbun, *Carcinus maenas* Linnaeus, *Pandalus borealis* Krøyer, *Nephrops norvegicus* Linnaeus, *Homarus americanus* Milne Edwards, *Orconectes virilis* Hagen, and *Calocaris macandreae* Bell. The two species on which the various extracts were tested by injection were *Palaemonetes vulgaris* and *Palaemon adspersus*.[2]

Particular glandular or ganglionic portions of eyestalks, ablated from light-adapted donors, were separated with the aid of a binocular dissecting microscope. Extracts of such tissues (whole eyestalks, sinus glands, sinusglandless eyestalks, specific optic ganglia, etc.) were prepared by triturating the pooled components with sand and sufficient solvent (sea water or distilled water) to give a final concentration of 10 units per 1.0 ml. for most of the tissues. The resulting tissue brei was heated in a boiling water bath for 2–3 minutes and centrifuged to remove extraneous material and coagulated proteins; in some cases the mixture was centrifuged without such heating and in still others comparison of activity was made between heated and unheated extracts of the same tissue. The particulars of such preparations are described for each donor species.

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[2] The vicissitudes of systematic nomenclature are frequently a source of confusion to experimental biologists. Several colleagues have called our attention to the revised synonomy of the genera with which we have worked. Dr. Fenner A. Chace, Jr. of the U. S. National Museum, Smithsonian Institution, has advised us that most of the European members of the genus *Leander* have been returned to the genus *Palaemon; L. adspersus* is therefore now referred to as *Palaemon adspersus*. Holthius (1952) has recently separated from *Palaemonetes vulgaris* of the Woods Hole region two additional species, *P. pugio* and *P. intermedius*. Examination of 100 *Palaemonetes*, randomly selected from animals furnished by the collectors, for differences in their rostral teeth, the character on which distinction between the three species can be most readily made, showed two *P. pugio* and no *P. intermedius*. The *Palaemonetes* in this study were therefore used without further regard to species distinction. Fingerman and Mobberly (1960) reported 8% *P. pugio* and 92% *P. vulgaris* in a similar examination at Woods Hole. They observed, in addition, no physiological distinction in retinal pigment responses between these two species.

The test and control animals, isolated in individual containers, were dark-adapted for 3 to 10 hours before injection. Each test animal was injected, at measured intervals, with 0.05 ml. of the prepared extract by the dim light from a red lamp. Uninjected control animals were exposed to the same light for comparable periods.

Since it has been shown (Kleinholz, 1936, 1938) that maximum response of dark-adapted distal retinal pigment cells both in *Palaemonetes vulgaris* and in *Palaemon adspersus* occurs between 30 and 45 minutes after injection of eyestalk extract, measurements in the tests with *Pandalus* and with *Nephrops* tissues were made in this interval; in all others the position of the distal retinal pigment of each test animal was measured 45 minutes after injection. This was done with a compound microscope furnished with an ocular micrometer. The two eyestalks of the prawn were ablated and oriented with the dorsal pigment spot uppermost in a small cell containing water. Two readings from the margin of the cornea were taken of each retina: one to the margin of the distal retinal pigment and the second to the level of the dorsal pigment spot (Fig. 1). From these readings the distal retinal pigment index was calculated (Sandeen and Brown, 1952). Such measurements were made within two minutes after removal of the eyestalks; this period of exposure to the light from the microscope lamp had no perceptible effect on the position of the distal retinal pigment.

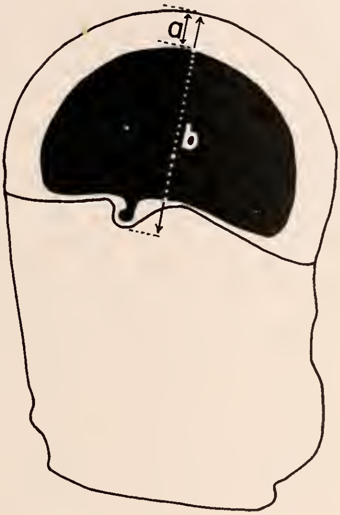

FIGURE 1. An eyestalk of *Palaemonetes vulgaris*, from the dorsal surface, showing the dimensions of the retina measured for calculation of the distal retinal pigment index, *a*/*b*.

Retinal pigment indices for the series of prawns injected with similar extracts were averaged and the standard deviations calculated for each series to show the variance among the results. Student's t distribution was used in finding the

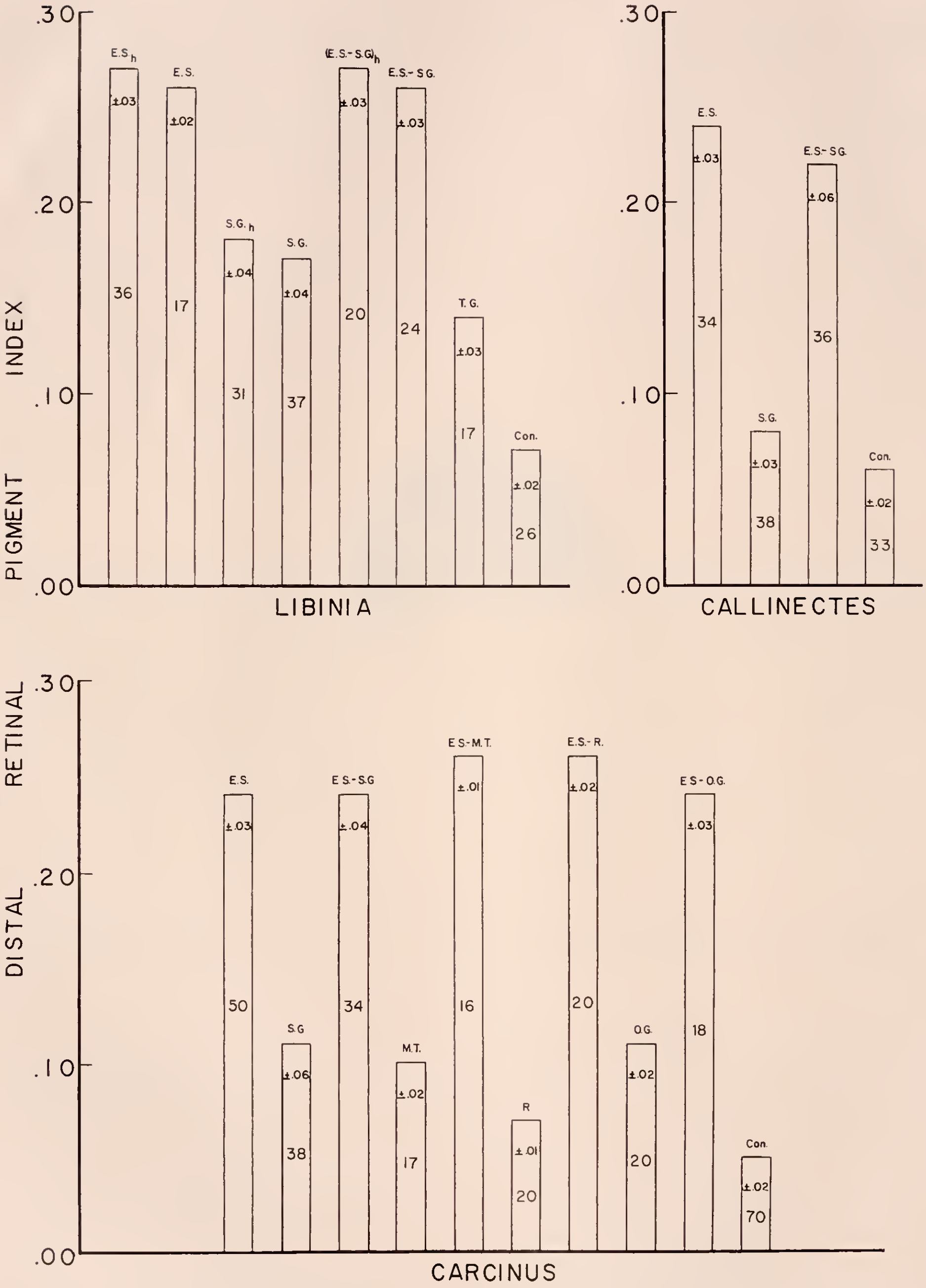

FIGURE 2. Distal retinal pigment light-adapting hormone in brachyuran nervous tissue, as shown by the effects of injected extracts on the degree of distal retinal pigment light-adaptation in test *Palaemonetes*. *ES*, extract of whole eyestalk; ES_h, extract of whole eyestalks, heated briefly in a water-bath; *SG*, sinus glands; SG_h, heated extract of sinus glands; *ES–SG*,

statistical significance between average distal retinal pigment indices of experimental series.

OBSERVATIONS

A. Brachyura

1. *Carcinus maenas*

This study was begun with extracts prepared from eyestalks and eyestalk components of *Carcinus maenas* and tested at Woods Hole on the dark-adapted retina of *Palaemonetes.* The donor *Carcinus* were light-adapted males and averaged 5.4 cm. in maximum carapace width. Tissues, in concentrations of 10 units per 1.0 ml. of sea water, were triturated, as described above; the supernatant was injected in 0.04-ml. doses into the abdominal musculature of each dark-adapted test animal.

The distal retinal pigment indices of test animals injected with these extracts are summarized in Figure 2. Extracts of sinus gland produced retinal pigment indices higher than those for the uninjected control *Palaemonetes* (0.11 as compared with 0.05) but much lower than those of test animals injected with extracts of entire eyestalks (0.24). Extract prepared from sinusglandless eyestalks, however, resulted in responses as marked as those given by extracts prepared from entire eyestalks. In similar fashion, extracts of medulla terminalis, of retina, and of the second and third optic ganglia, each produced some degree of light-adaptation of the distal retinal pigment as evidence for the presence of retinal pigment hormone in the tested extract. Extracts of the remainder of the eyestalk from which these particular components had been removed evoked responses similar to that produced by the entire eyestalk.

2. *Callinectes sapidus*

In an earlier account (Kleinholz, 1936) the slight effect of *Callinectes* eyestalk extract on the distal retinal pigment of dark-adapted *Palaemonetes* was complicated by high mortalities among the test animals. In the present study, eyestalks were removed from light-adapted male crabs, averaging 12.4 cm. in carapace width, measured from tip to tip of the lateral spines. Extracts in concentrations of 6 units (*i.e.* whole eyestalks, sinus glands, etc.) per 1.0 ml. of sea water were used without untoward effects on the survival of the injected test animals if the crude extract was heated for two minutes in a boiling water bath, and the coagulated proteins removed by centrifugation.

It is apparent from Figure 2 that injected sinus gland extracts cause only slight light-adaptation (the retinal pigment index is 0.083 compared with the

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eyestalks from which the sinus glands had been removed; $(ES\text{–}SG)_h$, heated extract of sinusglandless eyestalks; *MT*, medulla terminalis or fourth optic ganglion; *ES–MT*, eyestalks from which medulla terminalis had been removed; *R*, retina, including portions of the lamina ganglionaris or first optic ganglion; *ES–R*, eyestalks minus the retinal region; *OG*, the second and third optic ganglia, not including the sinus gland; *ES–OG*, eyestalks, including the sinus glands, but minus the second and third ganglia; *TG*, thoracic ganglia; *CON*, control, uninjected dark-adapted test animals, exposed briefly to the light from a red photographic lamp. The numbers near the top of each bar are the standard deviations (rounded to the nearest hundredth) from the average distal retinal pigment index. The numbers near the middle of each bar indicate the number of retinas measured in calculating the average index.

0.057 of the uninjected controls) whereas extracts of eyestalks without the sinus glands produce a response almost that obtained with extracts of whole eyestalks (indices of 0.219 and 0.235, respectively).

3. *Libinia emarginata*

Nervous tissues, taken from light-adapted males weighing about 500 grams, were ground and extracted with sea water to a final concentration of 10 units per 1.0 ml. One set of extracts of whole eyestalks, of sinus glands, and of eyestalks without sinus glands was heated for two minutes at 100° C., and the activity in distal retinal pigment hormone compared with that of the like series of unheated extracts. The fused mass constituting the thoracic ganglion also was extracted for injection. Since the wet weight of such a ganglionic mass is approximately equal to that of the soft tissue of an eyestalk from the same animal, extracts of thoracic ganglia were prepared in concentrations of 10 per 1.0 ml.

The results of tests with such tissue extracts from *Libinia* are shown in Figure 2. Sinus gland extracts, although producing substantial migration of the distal retinal pigment in the injected test animals (Retinal Pigment Index = 0.166), are appreciably less active than extracts of entire eyestalks (RPI = 0.261) or extracts of sinusglandless eyestalks (RPI = 0.255). Thoracic ganglia extracts produce a lesser degree of light-adaptation of the distal retinal pigment cells (RPI = 0.138) than do the other tissue extracts. The retinal pigment indices effected by injection of heat-treated extracts (RPI for whole ES = 0.268; RPI for SG = 0.178; RPI for ES–SG = 0.265) are not significantly different from those produced by the unheated extracts.

In the three species of crabs examined, the activity of retinal pigment hormone in sinus gland extracts is consistently less than in extracts of sinusglandless eyestalks. The differences in degree of light-adaptation of the distal pigment cells produced by sinus gland extracts from these brachyurans have not been examined more closely; they may be due to size differences between the donor species, or to the number of sinus glands used in preparing the extracts.

B. Caridea [3]

1. *Pandalus borealis*

Similar studies with comparable extracts prepared from the eyestalks of decapod crustaceans other than brachyurans seemed desirable, and advantage was taken of an opportunity to make such tests with the relatively large eyestalks of *Pandalus* at the Kristineberg Zoological Station. *P. borealis* is a protandrous hermaphrodite whose biology has been described by Rasmussen (1953) and by Horsted and Smidt (1956). Carlisle (1959) has reported on the histology of the neurosecretory elements in the eyestalk of *Pandalus*. The components of the eyestalk tested for retinal pigment hormones are shown in Figure 3.

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[3] These studies with tissue extracts of *Pandalus borealis* were made in 1957 in collaboration with Dr. David B. Carlisle at the Kristineberg Zoological Station in Sweden. D. B. C. dissected and separated the various eyestalk components of *Pandalus;* activity of the extracts was tested by L. H. K. and D. B. C. We are indebted to Dr. G. Gustafson, then director of the Station, for making laboratory accommodations available to us for this study.

Extracts of such components, either singly or in various combinations, were prepared in concentrations of 10 units per 1.0 ml. of sea water and heated for two minutes at 85° C. Similar preparations of sinus glands and of eyestalks minus sinus glands, without such heating, were tested in comparison with the heat-treated extracts. In addition, extracts of 10 ventral nerve cords (from telson to circumesophageal connectives) per 1.0 ml., of walking legs and swimmerets for control tests, and of eyestalk preparation of erythrophore-concentrating hormone, generously given us by Drs. Östlund and Fänge, were tested for retinal pigment activity.

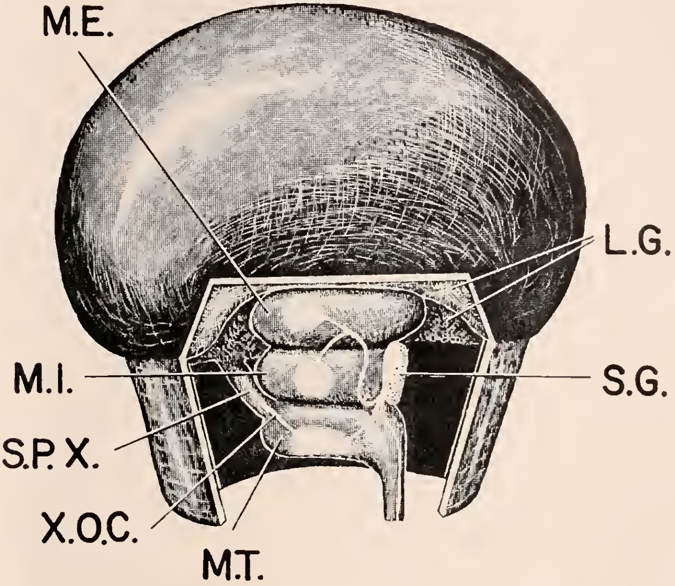

FIGURE 3. Dissection of a left eyestalk of *Pandalus borealis*, approaching somewhat obliquely from the dorso-abaxial aspect, so that the mid-dorsal line is towards the right-hand side of the drawing. Only the nervous tissues are shown. Ganglionic X-organs show in their natural appearance as whitish patches against their corresponding medullae; from each of them a neurosecretory tract runs to the sinus gland. A tract also runs from the brain to the sinus gland. In life these tracts also appear as white lines against the nervous tissue. Dissection by D. B. Carlisle. *LG*, lamina ganglionaris; *ME*, medulla externa, or second optic ganglion; *MI*, medulla interna; *MT*, medulla terminalis; *SG*, sinus gland; SPX, sensory pore X-organ (Hanström's X-organ); *XOC*, X-organ connective.

The unavailability of *Palaemonetes vulgaris* at Kristineberg necessitated the use of *Palaemon adspersus* as the test animal. The responses of the distal retinal pigment of dark-adapted *Palaemon adspersus*, measured between 30–45 minutes after injection of various concentrations of eyestalk extracts (Kleinholz, 1938), are similar to those shown by *Palaemonetes*, in that the responses are graded according to concentration. The distal retinal pigment indices, however, are slightly different from those in *Palaemonetes*. In dark-adapted, uninjected *Palaemon* the distal retinal pigment index averaged 0.029, while that for animals light-adapted for three hours in direct sunlight averaged 0.257.

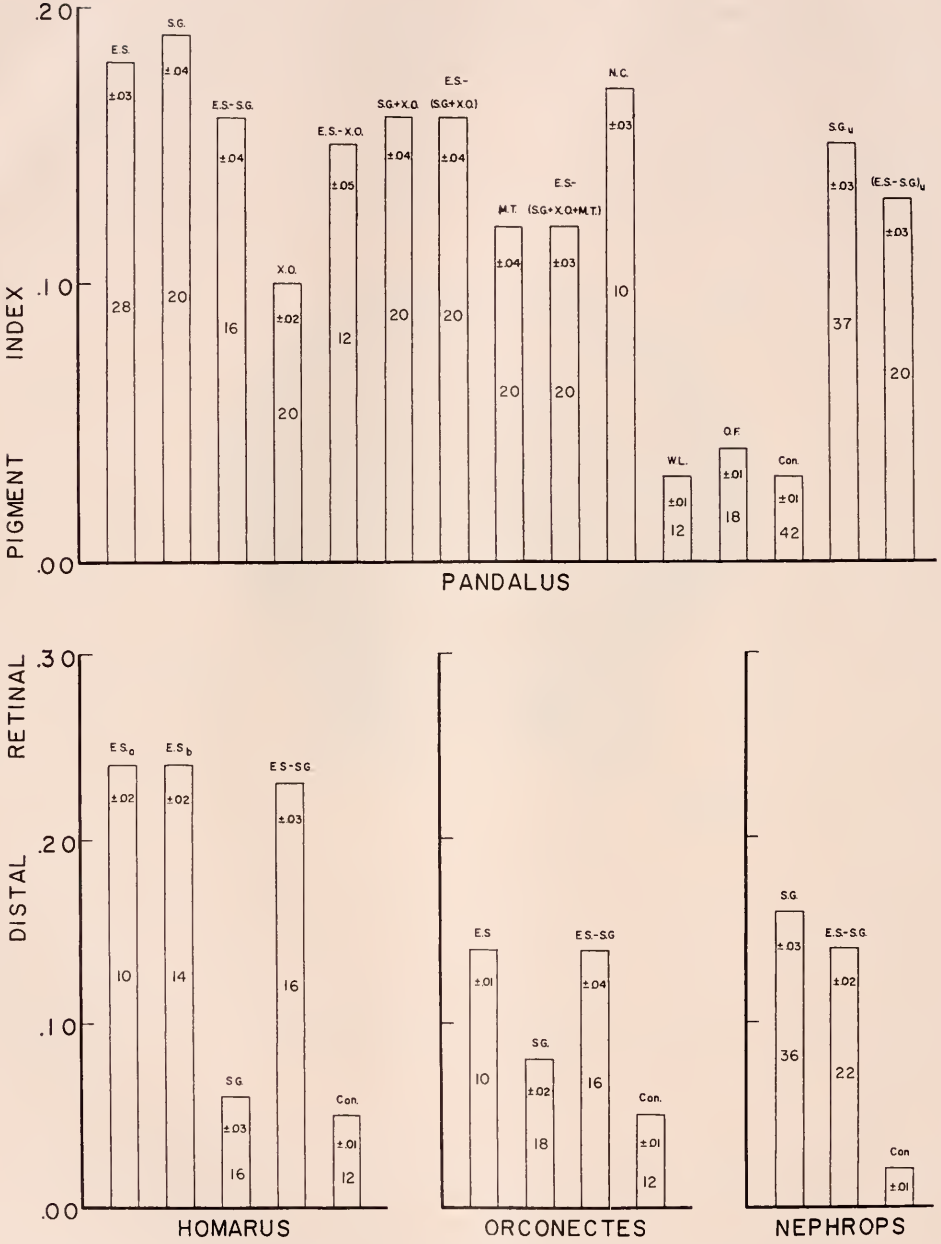

FIGURE 4. Distal retinal pigment light-adapting hormone in caridean (*Pandalus*) and macruran nervous tissues. Extracts of *Pandalus* and of *Nephrops* tissues were tested on *Palaemon adspersus;* tissues from *Homarus* and from *Orconectes* were tested on *Palaemonetes. ES*, extract of whole eyestalks; ES_a, extracts of whole eyestalks of *Homarus*, in a concentration of 6 ES per 1.0 ml.; ES_b, extracts of whole eyestalks of *Homarus*, in concentration of 3 ES per 1.0 ml.; *SG*, sinus gland; SG_u, unheated sinus gland extract from *Pandalus; ES–SG*, sinus-

The results of these tests with *Pandalus* tissues are summarized in Figure 4. Several interesting features are apparent in this summary. First, extracts of *Pandalus* sinus gland, in contrast with brachyuran sinus gland extracts, effect as much light-adaptation in the test animals as do extracts of whole eyestalks (distal retinal pigment indices of 0.191 and 0.175, respectively). Second, extract of eyestalks from which sinus glands have been removed, as with the brachyurans, also produces high distal retinal pigment indices, the average being 0.159. Third, extracts of ventral nerve cord contain light-adapting hormone, the average distal retinal pigment index being 0.172. Fourth, as occurred also in tests with *Carcinus* tissue, extracts prepared from components of *Pandalus* eyestalk are effective in bringing about various degrees of light-adaptation of the distal retinal pigment. Fifth, the erythrophore-concentrating hormone preparation of Edman, Fänge and Östlund (1958), which we tested and found active on the small erythrophores of destalked *Palaemon adspersus* in a concentration of 1 : 100,000, was only slightly effective in causing light-adaptation in a concentration of 1 : 10,000, the resulting distal retinal pigment index being 0.040. Sixth, the differences in distal retinal pigment index between heated extracts (of sinus gland, 0.191; of eyestalk minus sinus gland, 0.159) and similar unheated extracts (of sinus gland, 0.148; of eyestalk minus sinus gland, 0.127) are significant. The difference for the sinus gland extracts is significant at the 0.001 level, that for the sinusglandless eyestalk extracts is significant at the 0.05 level but not at the 0.01 level.

C. Macrura

1. *Nephrops norvegicus*

Eyestalk tissue of *Nephrops norvegicus* was tested on *Palaemon adspersus*, but because of the limited availability of this donor species at the time, extracts of sinus glands and of eyestalks minus sinus glands were prepared. These extracts were unheated, and in concentrations of 6 units per 1.0 ml. of sea water. The distal retinal pigment indices produced by the two kinds of extracts are similar to those indices resulting after injection of comparable tissue extracts prepared from *Pandalus* (Fig. 4).

2. *Calocaris macandreae* [4]

This species is a blind, mud-burrowing form; its eyestalk is rudimentary and possesses no retina. A sinus gland, however, is present and when injected in aqueous extract into test *Palaemon adspersus* causes concentration of erythrophore pigment (Carlisle, unpublished observations).

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[4] D. B. C. removed the tissues and prepared the extracts. Retinal pigment hormone activity was tested jointly with L. H. K.

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glandless eyestalks; $(ES\text{–}SG)_u$, unheated extract of sinusglandless eyestalks from *Pandalus*; *XO*, Hanström's X-organ (*SPX* of Figure 3); *ES–XO*, eyestalks from which Hanström's X-organ had been removed; *SG* + *XO*, sinus gland and Hanström's X-organ; *ES*–(*SG* + *XO*), eyestalks from which sinus glands and X-organs had been removed; *MT*, medulla terminalis; *ES*–(*SG* + *XO* + *MT*), eyestalks with sinus gland, X-organ and medulla terminalis removed; *NC*, ventral nerve cord; *WL*, control extracts prepared from walking legs and swimmerets; *OF*, erythrophore-concentrating hormone preparation of Edman, Fänge, and Östlund; *CON*, control uninjected test animals.

Protocerebrum and optic stalk of *Calocaris* were triturated and extracted in distilled water, to give a final concentration of 10 per 1.0 ml., and the preparation heated for two minutes at 85° C. Injection of the supernatant into dark-adapted test *Palaemon* resulted in an average distal retinal pigment index of 0.032 ± 0.007 (standard deviation) for 18 eyestalks; the average index for uninjected control *Palaemon* was 0.027. A more concentrated extract, 80 units per 1.0 ml., prepared in sea water and heated as before gave an average retinal pigment index of 0.078 for 20 test eyestalks, with the index for the uninjected control animal being 0.042.

3. *Homarus americanus*

The interesting differences between sinus glands of brachyurans and those of *Pandalus* and of *Nephrops* in their effects on distal retinal pigment migration made tests with additional macruran species desirable. Eyestalks of light-adapted *Homarus* were triturated in measured amounts of sea water and the brei heated for two minutes at 95° C. Extracts of whole eyestalks, prepared in concentrations of 6 eyestalks per 1.0 ml. and of 3 eyestalks per 1.0 ml., were centrifuged and 0.04 ml. of supernatant injected into test *Palaemonetes*. Extracts of sinus gland and of eyestalk minus sinus gland, each in concentrations of 3 units per 1.0 ml., were similarly prepared and injected.

No difference in the distal retinal pigment index of test animals is observable between the two concentrations of whole eyestalk extract (Fig. 4). Sinus gland extract, on the other hand, shows relatively little effect on the dark-adapted distal retinal pigment, being similar to brachyuran sinus gland extract in this respect. Extract of sinusglandless eyestalks shows substantial activity in light-adapting retinal pigment hormone.

4. *Orconectes virilis*

Light-adapted male crayfish, between 40–42 mm. in rostrum-carapace length, served as donor animals. Eyestalk tissues were triturated and distilled water added to give final concentrations of 10 units per 1.0 ml. The unheated extracts were centrifuged and 0.05 ml. of the supernatant injected into each dark-adapted test *Palaemonetes*.

The summarized results (Fig. 4) show that extracts of whole and of sinusglandless eyestalks produced the same degree of light-adaptation of the distal retinal pigment in the test animals. Sinus gland extracts effected substantially less light-adaptation. The distal retinal pigment indices resulting from these winter tests with crayfish eyestalks are lower than those obtained with extracts of brachyuran eyestalks.

DISCUSSION

The tests used in this study are not quantitative assays of the amount of light-adapting distal retinal pigment hormone in a particular extract. The distal retinal pigment index has served predominantly as an indicator that extracts of whole eyestalks were near the upper threshold in concentration and were effecting near-maximum response of the distal retinal pigment. Thus, a high distal retinal pigment index obtained with a certain concentration of eyestalks establishes a reference response. If a retinal pigment index considerably lower than this reference results from tests with identical concentrations of sinus gland, a low hormone content is indicated. If, however, such sinus gland extracts result in near-maximum retinal pigment indices, a high hormone content of the sinus glands is indicated.

A qualification might be made to this latter conclusion. If a particular concentration of whole eyestalks were very much higher than the upper threshold concentration, tests with extracts containing the same number of sinus glands might then result in responses that approached or equalled the maximum. This contingency could be avoided by a knowledge of previously established concentration-response curves constructed for each of the tissues to be tested. Such an assay curve, with *Palaemonetes* being used as the test animal, has been made for *Libinia emarginata* (Kleinholz, unpublished). It was found that eyestalks of *Libinia* in concentrations of 2 per 1.0 ml. cause maximum light-adaptation of the distal retinal pigment. The concentrations of 10 units per 1.0 ml. for the *Libinia* tests shown in Figure 2 are therefore about four to five times the upper threshold concentration, yet the distal retinal pigment index resulting from injecting sinus gland extracts of this concentration is considerably below that for extract of whole eyestalks. Another explanation of the differences in distal retinal pigment index obtained with extracts of *Pandalus* sinus gland and those of brachyuran sinus gland might be that of differences in sensitivity of the test *Palaemon* and *Palaemonetes* to light-adapting hormone. Extracts of sinus glands from *Carcinus maenas* of Woods Hole, when tested on *Palaemonetes*, effect low retinal pigment indices; when tested in the same concentration on *Palaemon adspersus* at Kristineberg they also produce low distal retinal pigment indices (Kleinholz, 1961). *Palaemon adspersus*, as a test species, is therefore not more sensitive to distal retinal pigment light-adapting hormone than *Palaemonetes*, and the differences in response produced by *Pandalus* and brachyuran sinus glands seem to be explained by the lower hormone content of the sinus gland among the species of the latter group.

The occurrence of distal retinal pigment light-adapting hormone in many parts of the central nervous system is in agreement with demonstrations (Bliss and co-workers, 1952, 1954; Carlisle, 1959; Sandeen, 1950) of neurosecretory cells and physiologically-active chromatophorotropins in these tissues. The marked responses of red chromatophores of *Palaemon adspersus* to extracts of sinus gland from *Calocaris* and to the hormone preparation of Edman, Fänge and Östlund, while such extracts show little activity on the distal retinal pigment, may support the possibility that two different hormones are involved in regulating these effectors. Comparable responses by the retinal and the integumentary effectors to brachyuran sinus gland extracts may imply, in addition, the terminal localization of distal retinal pigment hormone in nervous tissue other than the sinus gland. Cytological studies now in progress may furnish additional information on this point.

The effect of heat-treatment on the activity of tissue extracts subsequently tested on distal retinal pigment cells cannot be commented on extensively at this time. With *Libinia* tissues the responses to heated and unheated extracts are not significantly different, whereas with *Pandalus* tissues they are. An enzyme that inactivates distal retinal pigment light-adapting hormone is present in several crustacean tissues (Kleinholz, unpublished). The differences between the two species cited have not been investigated but may be due, among other possibilities, to variations in manipulative detail before the enzyme in the tissue extracts was denatured by the heat treatment.

Conclusions

1. Distal retinal pigment light-adapting hormone can be demonstrated in a number of crustacean nervous tissues and is in agreement with the histological demonstration of neurosecretory cells and axons in such tissues.

2. Among crustacean species, differences may be found in the activity of sinus gland extracts on the retinal pigment effectors. The basis for these differences is not yet known.

3. Heated extracts of *Libinia* tissues result in distal retinal pigment indices not significantly different from those obtained with unheated extracts. With similarly treated *Pandalus* extracts the differences in distal retinal pigment index are significant.

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GAMETOGENESIS AND SPAWNING OF THE EUROPEAN OYSTER, O. EDULIS, IN WATERS OF MAINE

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In the late '40's a heavy mortality of an epizootic nature occurred among the populations of the soft clam, *Mya arenaria*, along the New England coast. The mortality was especially serious in the waters of Maine where, in many areas, these clams almost completely disappeared. Since *M. arenaria* was virtually the only commercial mollusk of that state, many shore communities were seriously affected economically. Realizing that economy established on a single shellfishery is insecure, we suggested that the local shellfisheries resources be supplemented by the introduction of another mollusk of commercial promise. We had in mind the European oyster, *Ostrea edulis*, which propagates at a somewhat lower temperature than our native oyster, *Crassostrea virginica*.

The European oysters were shipped to Milford from the Oosterschelde, Holland, in the fall of 1949. Large oysters arrived in good condition and, after resting in Milford Harbor, were planted in Boothbay Harbor and three other locations in Maine (Loosanoff, 1951, 1955). In Boothbay Harbor the oysters grew well and showed comparatively low mortality. Gonadal samples, taken from this group at regular intervals, provided the material for the histocytological studies on which the present paper is based. The tissue was preserved in Bouin's fluid, sectioned at 5 μ, and stained with Heidenhain's iron hematoxylin and eosin.

A description of the sexual phases of the European oyster is not the purpose of this article. This matter has been considered in a number of extremely informative publications, starting with Hoek (1883); Spärck (1925); and, especially, Orton (1927, 1933). Later it was reviewed by Coe (1936). Obviously, it is not necessary to go into the details of this complex problem again. Therefore, this article describes only two important aspects of the behavior of European oysters—gametogenesis and spawning under the new set of ecological conditions encountered in Boothbay Harbor.

It may be appropriate to mention here, chiefly as a reminder, Orton's discovery that the European oyster is protandric, with the sexual phases alternating regularly, in most individuals, after the initial male phase. Under favorable conditions each adult oyster completes one male and one female phase each year. Some oysters function as males early in the spawning season and later change to the female state. Others have the opposite sequence of phases. Because of this situation, individuals of both sexual types are found in the population during the entire spawning season (Cole, 1941).

Orton (1927) also suggested dividing the oysters into approximately eight arbitrary categories based upon the relative numbers of cells of different sexes in their gonads. These categories, which were established principally on studies of gonadal smears, do not, however, appear to be precise enough if compared to results of similar studies in which regular histological preparations are used. The latter, based on a large number of sections, would show that different portions of a gonad of the same individual may often give an entirely different characterization of the sexual conditions of that individual. In other words, while some portions of the gonadal tissue of an individual may characterize it, according to Orton's standards, as a pure male, another and often closely adjacent portion of the gonad may display purely female conditions. Sometimes, in the same individual the adjacent follicles might contain cells of opposite sexes although the individual, as a whole, is, obviously, ambisexual.

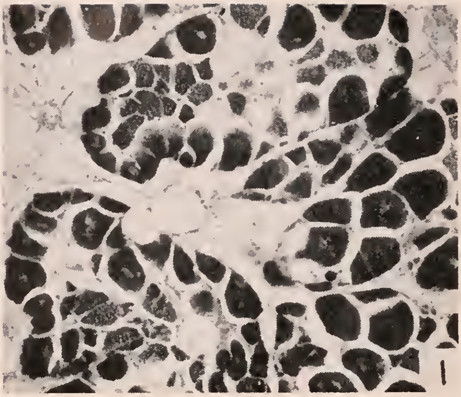
Since virtually all of the Boothbay Harbor oysters studied were ambisexual to some extent, we hesitate to accept the classification of Orton, who assumed that there are pure male and pure female individuals which, presumably, contain exclusively male or female cells, respectively. Accordingly, in this study we shall designate all individuals as representing three chief categories, namely, (a) strongly ambisexual (Fig. 1); (b) predominantly male (Fig. 2); and (c) predominantly female (Fig. 3).

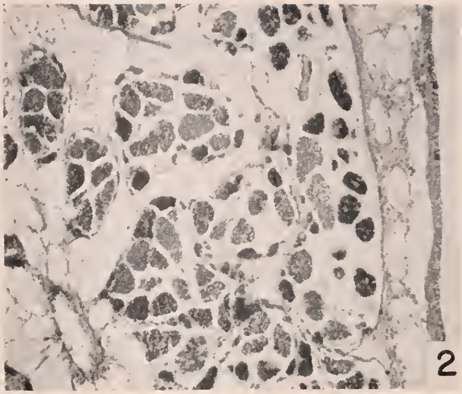
It is convenient for several reasons to begin the discussion of the seasonal sexual changes of the European oysters kept in Boothbay Harbor with the spring gametogenesis. Although in some instances it may begin in April, the population in general does not display early stages of gametogenesis until the latter part of May. Even at that time, approximately 10% of the oysters still possess typical winter follicles, which are small, contracted and surrounded by large masses of connective tissue (Fig. 4). At this time they contain only indifferent cells or gonia, although phagocytic cells are often present in the lumina.

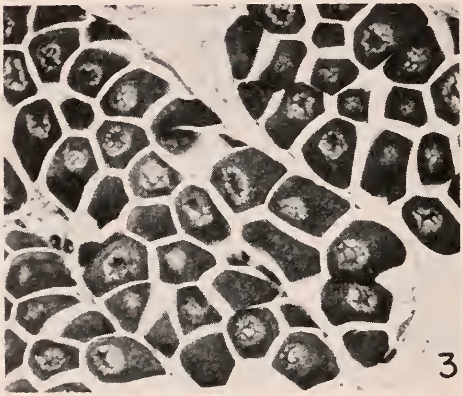
In oysters already undergoing spring gametogenesis, follicles in the same individuals are often in widely different stages of development. Although, in general, they are still small and surrounded by connective tissue, which occupies most of the space between digestive gland and outside membrane of the oyster body, a few sperm-balls are already present in males. In the majority of cases, however, only spermatids or spermatocytes have been formed so far. At this period, developing eggs are usually small, measuring under 50 μ (Fig. 5).

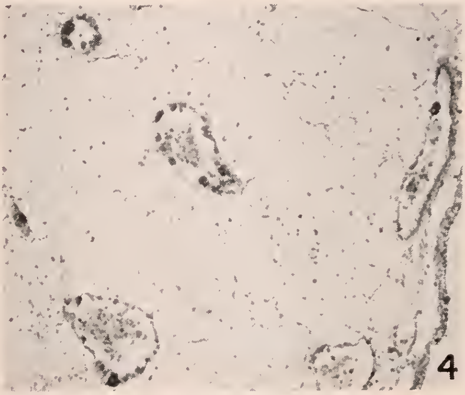
Two basic differences can be noticed between the general structure of the gonads of American and European oysters at this time and, also, later in the season. One is that the tissue layer for potential development of gonad material is significantly thicker in American than in European oysters of the same size. In the latter, the layer of gonadal tissue, *i.e.*, the area demarcated from the outside by the body wall and from the inside by the digestive gland, may very often consist of a single layer of follicles. Actual measurements show that while in American oysters 3″ to 4″ in size the thickness of gonadal layer may often be 3.5 to 4 mm., in European oysters of the same size it rarely exceeds 1.25 mm. Another basic difference is that while in American oysters the follicles are surrounded by a large number of leucocytes (lymphocytes) which probably act as carriers of nutritive material to the developing follicles (Loosanoff, 1942), cells of this type are entirely absent in *O. edulis* (Fig. 6).

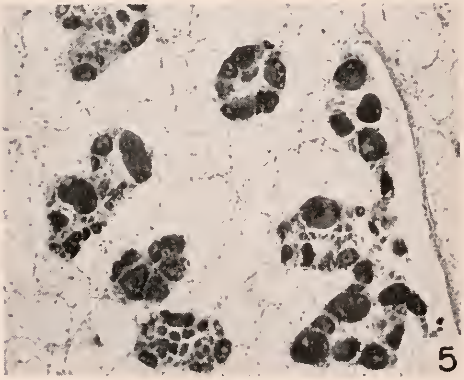
By the middle of June the gonadal layer is still comparatively thin and only in exceptional cases do proliferating follicles extend all the way to the digestive gland.











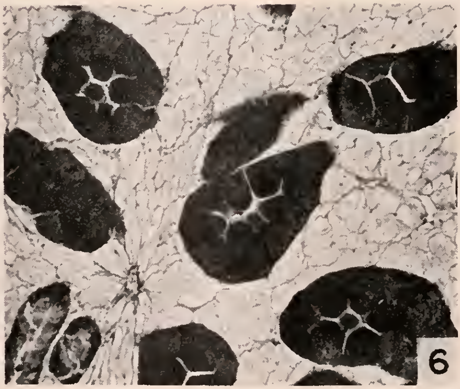


FIGURE 1. Ripe ambisexual gonad of *C. edulis.* Sperm-balls and groups of cells in earlier stages of spermatogenesis are in the center of the follicles. Note connections between blood vessels and apices of the follicles. × 80.

FIGURE 2. Ripe predominantly male gonad of *O. edulis.* Many sperm-balls can be seen in genital canal. × 80.

FIGURE 3. Ripe predominantly female gonad of *O. edulis.* × 80.

FIGURE 4. Winter follicles of *O. edulis* confined between body wall and digestive gland

Nevertheless, more than half of the follicles with male cells contain what appear to be fully developed sperm-balls (Fig. 7). Spermatids become more numerous while primary and secondary spermatocytes begin to decrease in numbers. Eggs measuring between 75 and 100 μ are already found in some individuals, but there is no evidence of spawning.

At the end of June and during the first week of July sperm-balls can be found in the genital canals of some oysters, yet no mass spawning has occurred.

Spawning begins during the second or third week of July and continues until about the end of August (Fig. 8). However, during the first part of this period many oysters are still unripe and gonadal follicles occupy only about half the available space. In many males, even those which have discharged some sperm-balls, large groups of spermatids, and even spermatocytes, are still present.

Toward the end of July unripe oysters are still common but in the group representing only 5 to 10% of the population, spawning is already completed and resorption of gonads is beginning. In the latter individuals shrinking of follicles is in progress and invasion of connective tissue is rapidly taking place. Most of the individuals that have completed spawning are males but, strangely enough, regardless of early completion of the male phase, there is no cytological indication that a female phase will follow. It is probable, therefore, that in many instances, because of our short, cold summers these oysters can complete only one reproductive phase each season.

By the middle of August approximately 75% of the oysters are either partially (Fig. 9) or completely spawned. The majority of the completely spawned individuals, however, are predominantly males. As observed earlier in the summer, it is apparent that the male phase would not be followed by the completed female phase. Only in exceptional cases are small ovogonia or ovocytes seen developing along the walls of the follicles but, due to the lateness of the season, their development cannot progress very far before the onset of winter and relative inactivity.

After completion of the major spawning activities, the rest of the period of comparatively high temperature, confined chiefly to September (Fig. 15), is characterized by resorption of gonads and accumulation of glycogen. Resorption is brought about principally by phago-leucocytic cells which enter the follicles not through the follicular walls, as in American oysters (Loosanoff, 1942), but directly through the blood vessels (Figs. 10 and 11). Accumulation of glycogen is demonstrated by development of masses of connective tissue containing it, which rapidly fills almost the entire area between the digestive gland and the body wall of the oyster. The follicles, situated as islands within this tissue, are small, contracted and often filled with phagocytic cells (Fig. 12).

Strangely enough, simultaneously with completely spawned individuals there are still apparently normal oysters that have undischarged sex cells. These vary from those that retain only few cells in the follicles, or at least in genital canals, to those that carry virtually an undischarged supply of gametes. These individuals

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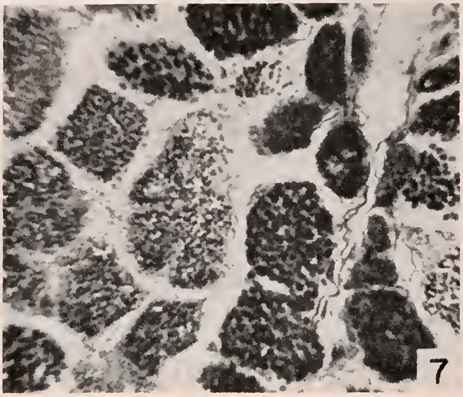
and surrounded by large areas of connective tissue. × 80.

FIGURE 5. Ambisexual gonad in early stages of spring gametogenesis. × 80.

FIGURE 6. Predominantly female gonad during spring gametogenesis. Note absence of leucocytes (lymphocytes) outside of follicular wall, and connections between blood vessels and follicles. × 80.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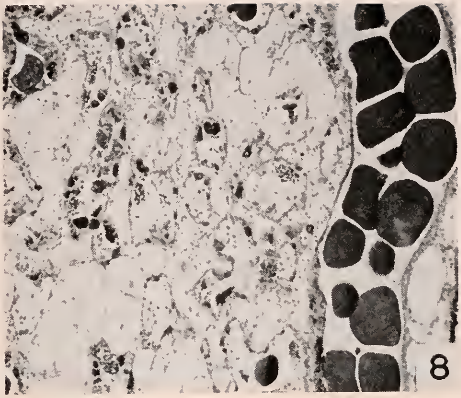
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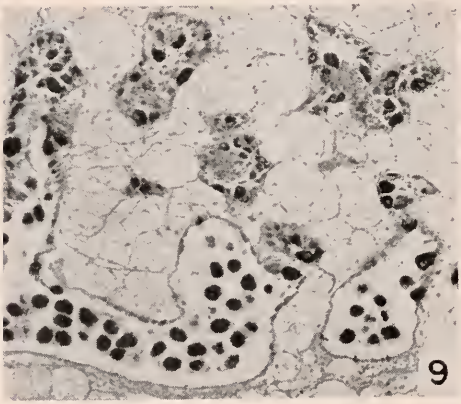
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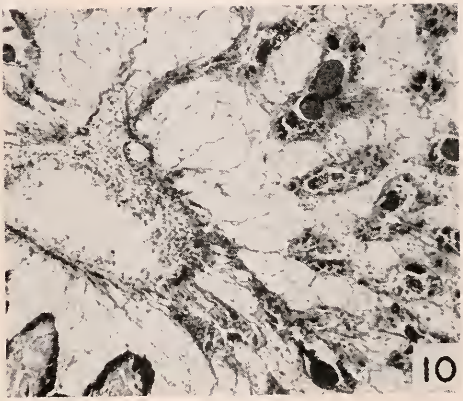
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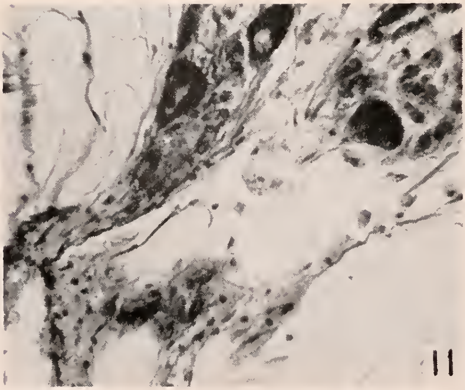
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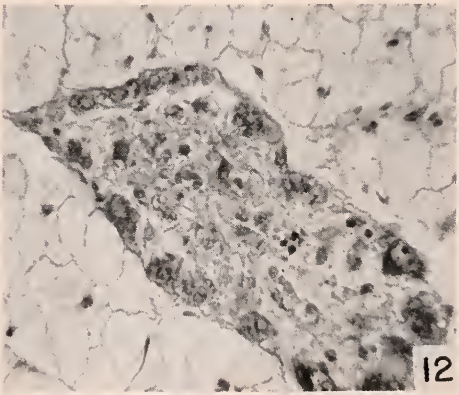
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FIGURE 7. Portion of male follicles showing groups of cells in different stages of development. Sperm-balls, characterized by presence of tails of spermatozoa, are seen in the center. × 345.

FIGURE 8. Ambisexual gonad during last stages of spawning. Remnants of male cells and some undischarged eggs can be seen in shrunken follicles, while a number of ripe eggs in the process of discharge are concentrated in the genital canal. × 80.

may belong to all categories, including both strongly defined groups, *i.e.*, those predominantly males and those that are almost pure females.

A still more confusing aspect of the situation is that in some individuals with definitely male characteristics, spermatogenesis appears to be still in progress. Furthermore, in the same individuals, and sometimes even within the same microscopic field, follicles may be located side by side, some of which are completely discharged and already resorbed, others, only partly discharged, and the third group may contain male or female cells that seem to be undergoing healthy gametogenesis. Such situations can be encountered in samples collected by the end of October and, in a few instances, even later in the year.

According to Orton (1927) the proportion of females remaining unspent at the end of the summer in English waters varies from 0 to 5%. In our case, however, the proportion was significantly higher, in some samples exceeding 25%.

In discussing the fate of unspawned eggs, Orton (1927, p. 974) suggested that the oysters eventually dispose of these cells in two ways: some ova may be either retained in the gonad and become resorbed or "they may be included in egg-cysts and extruded in masses and excreted *en bloc* on to the internal face of the shell and covered over with nacreous or horny matter in the form of an excretion blister." While we agree with the first method suggested by Orton, we cannot accept his second suggestion which has not been supported by any reliable observations.

Striking differences in the condition of the gonad of the European oyster at the end of the spawning period in Boothbay Harbor probably suggest that we did not deal with a homogeneous population, but a group composed of representatives of physiologically-different races. Loosanoff and Engle (1942) and Loosanoff and Nomejko (1951) have demonstrated the existence of such physiologically-different races of the American oyster, *C. virginica*, along our Atlantic coast. These races require different minimum temperatures for development of gonads and inducement of spawning. In general, the breeding temperature requirements of the northern oyster are lower than those of the southern group. Korringa (1957) came to a similar conclusion regarding European oysters. He stated that the general population of that species is composed of several distinctly different physiological races which require different temperatures to carry on normal propagation activities.

Since French seed oysters are sometimes imported for cultivation in Holland, it is quite probable that within the sample sent to us there were individuals the genetic complex of which was such that their temperature requirements for reproduction were higher than those of the true northern oysters of Holland. As a result, because of the short, cold summers of Boothbay Harbor, these oysters were unable

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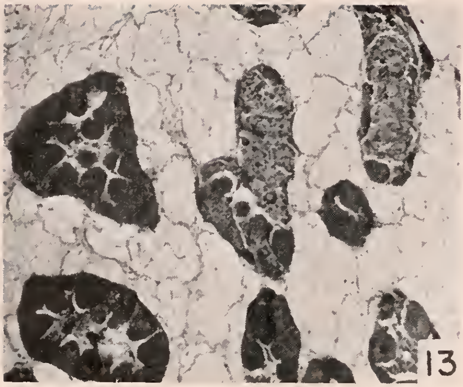
FIGURE 9. Partly discharged predominantly male gonad. Groups of sperm-balls are seen in the genital canal, while many follicles are contracting and are invaded by phago-leucocytic cells. × 80.

FIGURE 10. Resorption of gonad of *O. edulis* after spawning. Note shrinking follicles and connections between follicles and blood vessels. × 80.

FIGURE 11. Migration of phago-leucocytic cells from blood vessels into apices of follicles. × 775.

FIGURE 12. Follicles in last stages of resorption. Large group of phago-leucocytic cells occupying lumen. Young sex cells along follicular walls can be seen. × 345.



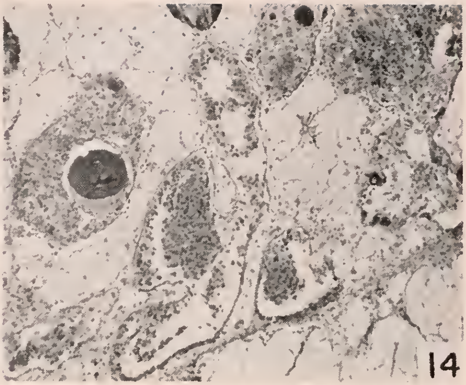


FIGURE 13. Cytolysis of predominantly female gonad that failed to ripen and be discharged. Note differences of degree of cytolysis of ovocytes in different follicles and also absence of phago-leucocytic cells outside follicular walls. × 80.

FIGURE 14. Advanced stages of resorption of predominantly female gonad. Follicles are filled with large numbers of phago-leucocytic cells. × 80.

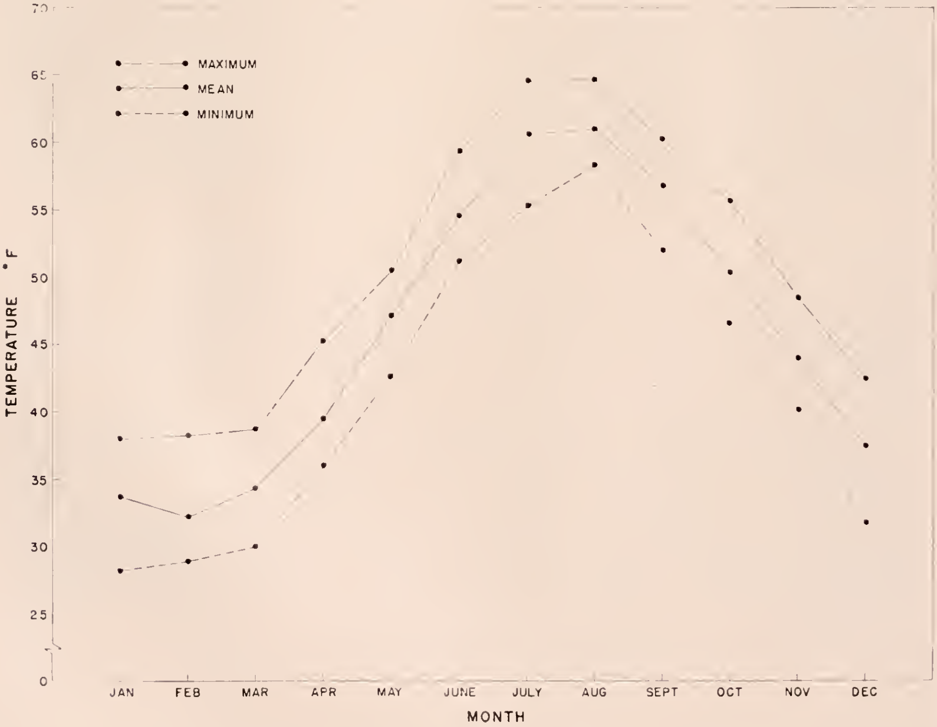


FIGURE 15. Mean, maximum and minimum monthly water temperatures at Boothbay Harbor, Maine during the period from 1906 to 1948.

to discharge their spawn. If the original shipment of European oysters, from which our samples for histological studies were collected, did include such races, it is quite probable that only the individuals carrying certain combinations of genes were able to propagate in Boothbay Harbor and are, therefore, responsible for the new population, which is now found in inshore areas of Maine (Welch, in press).

Studies of gonads collected late in November and December indicate that oysters can roughly be classified into two groups. In the first, resorption is almost or already completed. The follicles may be still full of phagocytes but, nevertheless, some young cells may be found along their walls. However, these cells are not differentiated.

The second group consists of individuals in which resorption of unspent sex cells is far from complete. Superficial examination may suggest that gonads are in the middle or at the beginning of the spawning period, but more detailed studies will show that cytolysis of undischarged cells, especially eggs, is in progress (Fig. 13). Thus, at this time of the year, as during other periods, there are pronounced individual differences between the condition of gonads of different oysters even if they belong, in general, to the same sex group.

Resorption of unspent cells continues through the winter. In some cases, it extends into April and perhaps even the first week of May, apparently depending upon individual conditions of the animals at the end of the preceding spawning season. However, at the same time and in the same oysters, in some of the follicles where resorption has already been completed, spring gametogenesis may be in progress. Consequently, two processes are going on simultaneously in the same individual—constructive gametogenesis and cytolysis. The latter is most active in female follicles where large groups of phagocytic cells attack remnants of the eggs (Fig. 14) which, in their appearance, greatly differ from each other, depending upon the stage of cytolysis.

As a rule, during early May no fully formed sperm-balls are found in the male follicles, but spermatids are occasionally seen. In female follicles normally developed ovocytes are clearly distinguishable, and in well advanced females, follicles may be seen proliferating almost to the digestive gland. In this way the annual sexual cycle of *O. edulis* is completed and begins anew.

These studies were made possible through the cooperation of Messrs. John B. Glude and Walter R. Welch, of the U. S. Bureau of Commercial Fisheries, who were kind enough to take care of the European oysters kept in Boothbay Harbor and send me preserved samples of gonads. Special thanks are due to Messrs. Richard E. Reed, former Commissioner, Sea & Shore Fisheries, Maine, and Dana E. Wallace, who were extremely helpful in overcoming a number of difficulties in connection with the introduction of *O. edulis* into this country.

SUMMARY

1. Active spring gametogenesis begins in May.

2. General spawning begins approximately during the second or third week of July and continues until about the end of August.

3. Because of short, cold summers, usually only one sex phase is completed by an individual oyster.

4. Resorption of gonads is carried principally by phago-leucocytes which enter the follicles not through the follicular walls, as in American oysters, but directly through the blood vessels.

5. Resorption of gonads may continue throughout the winter and early spring.

6. Differences in conditions of gonads of *O. edulis* planted in Boothbay Harbor suggest that the original group was not composed of a homogeneous population, but consisted of different physiological races.

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SUBLITTORAL ECOLOGY OF KELP BEDS OF THE OPEN COAST AREA NEAR CARMEL, CALIFORNIA

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The intertidal fauna and flora of the Monterey Bay area in central California are well-known, but the immediate subtidal associations, especially on the open coast where *Nereocystis* kelp predominates, are largely unexplored in places exposed to surf action. Free diving with the aqualung now makes possible direct observation of sublittoral zones (Drach, 1958).

METHODS

Diving along the open coast was undertaken in selected locations approximately nine miles south of Carmel, near Granite Creek (36° 26.5′ N. Lat., 121° 55.5′ W. Long.), between August, 1959, and September, 1961. This report is based on observations and collections made on 30 dives, amounting to approximately 20 hours underwater. Eleven dives were made during the summer of 1959, nine were made during the summer of 1960, and ten dives were made in alternate months during the fall, winter and spring of the two-year period of observation.

Diving from protected shore areas is possible in the summer during the calm periods associated with most low tides, but during the winter months continuous heavy swells make diving possible only on exceptionally calm days. After each dive, the collected material was identified and all observations recorded. During calm weather visibility may exceed 40 feet, but following rough weather or during phytoplankton blooms, it may be restricted to less than 10 feet. The profusion of forms found in sublittoral zones requires the diver to make many successive dives; only when most of the fauna is recognizable is it possible to note different patterns of distribution and to measure or estimate abundance.

OPEN COASTAL CONDITIONS

The coastal area from the Monterey Peninsula south to Point Sur and further south to San Simeon is characterized by steep granite cliffs (Fig. 1). Relatively deep water is found inshore; beaches are few and are usually limited to small coves. The shoreline is highly irregular. Uneven weathering of the granite has left pinnacles, some of which are submerged or constitute small islands. These small islands and projecting reefs afford protected areas in which the water between the open ocean and the shore may be 30 feet deep.

Upwelling is characteristic of this coastal area, producing cool temperatures, especially in the spring and summer. Monthly temperature readings have been made at Soberanes Point, one mile to the north of Granite Creek, by personnel of

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Hopkins Marine Station. Averages of monthly temperatures taken from 1956 through 1960 show a minimum of 10.8° C. in April and a maximum of 13.4° C. in September. The extremes over this five year period were 8.8° C. in June of 1959 and 14.6° C. in September, 1957. At Mussel Point in Monterey Bay, averages of daily readings for the same period reached a minimum of 12.5° C. in February and a maximum of 15.1° C. in September. These readings are approximately two degrees higher than on the outer coast to the south where upwelling is prevalent. Salinities in the Monterey Bay area usually vary between 33.5‰ and 34.0‰ depending on the season and rainfall.

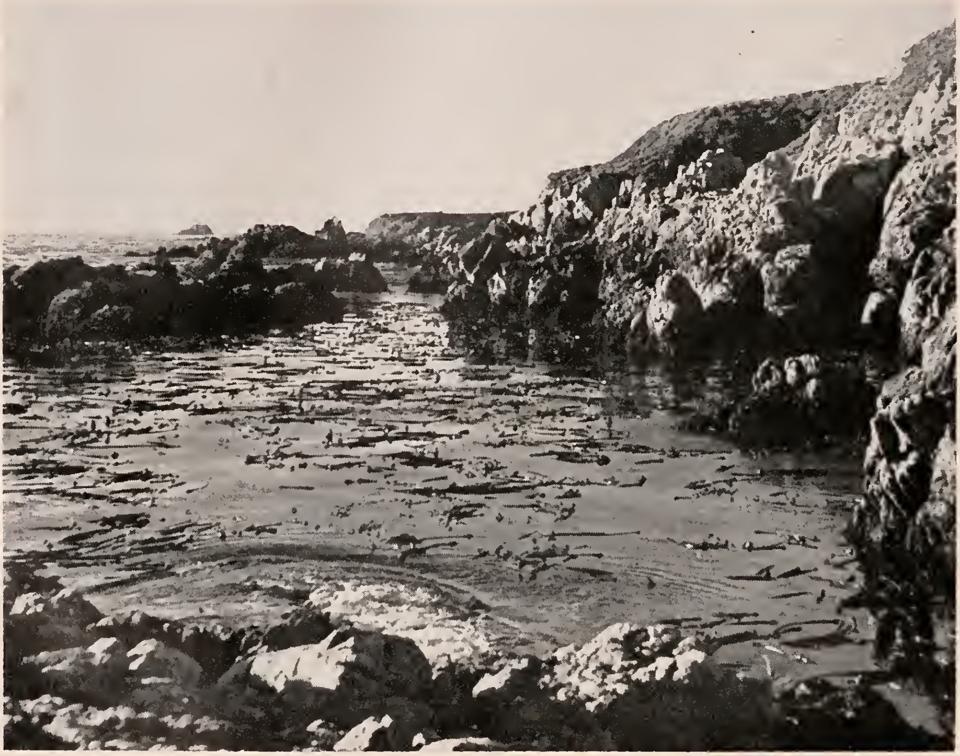

FIGURE 1. The diving area at Granite Creek, looking north. Inshore walls are steep and the small islands afford protected diving areas. *Nereocystis* kelp, shown at the surface, is anchored in depths of 40 feet.

The major kelp along the open coast in the Monterey Bay area at depths of 15 to 60 feet is the bulb kelp, *Nereocystis luetkeana*, which develops best in exposed conditions (Hurd, 1916). The *Nereocystis* beds extend only to a distance of 100 to 200 yards offshore where depth rapidly increases. Large stands of the giant kelp, *Macrocystis pyrifera*, are found in the more sheltered areas of Monterey and Carmel bays and south of Point Sur. Occasional plants occur along the open coast in the Monterey Bay area. The *Macrocystis* beds of southern California usually occur well offshore from beaches and are of greater width.

The stipe of *Nereocystis* may reach a length of 60 feet. Blades are produced only on the terminal float. I found that a typical plant has approximately 72 blades, each of which is three inches wide and up to eight feet in length. The blades of a single plant may have an area up to 70 square feet (on one side), hence a very dense surface canopy is produced where *Nereocystis* is aggregated. The holdfast is relatively small, reaching a diameter of only six inches. The stipes are about three-eighths of an inch in diameter in their basal portions. A cable of 10 to 20 stipes twisted together is usually formed where the plants grow thickly.

Nereocystis is an annual plant. Large quantities of this kelp are cast ashore on beaches during the fall months. The surface canopy is usually lost by late December, although a few plants persist through January. Young plants about a foot in height can be seen in April. In June the surge and tidal currents twist the stipes together before many of them reach the surface. During the late spring, summer, and early fall, the *Nereocystis* canopy exerts a profound shading effect upon its environment.

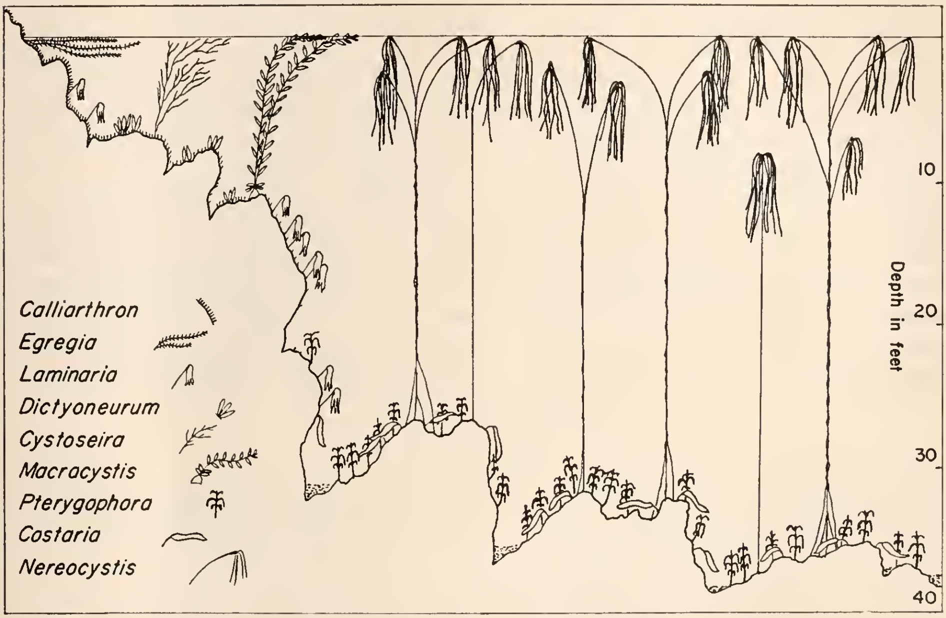

FIGURE 2. Diagrammatic cross-section of the *Nereocystis* kelp bed at Granite Creek, showing zonation of major algae. Depths are in feet below mean low water. The horizontal scale is approximately the same.

Intertidal forms are those which characterize the open coast environment described by Ricketts and Calvin (1952). The alga *Postelsia palmaeformis* receives the full force of the surf on rocky points; beds of *Mytilus californianus* are found somewhat lower, and lower still, extensive beds of the purple sea urchin, *Strongylocentrotus purpuratus,* are observed. At about the mean low-tide level in areas protected from the full force of the surf, the ribbon kelp, *Egregia menziesii,* produces a floating canopy extending ten or more feet out. The blades are annual; when present, this kelp has a significant shading effect upon the immediate subtidal environment.

THE CALLIARTHRON ZONE

The immediate subtidal zone, down to a depth of over ten feet, usually has a dense growth of a branching red coralline alga, *Calliarthron cheilosporioides* (Fig. 2). In southern California and in the more protected areas of central California, such as Monterey Bay, this zone is dominated by the eel grass, *Phyllospadix scouleri*. On level surfaces within this zone, a small kelp, *Dictyoneurum californicum,* covers large areas. The brown alga *Cystoseira osmundacea* grows thickly at depths to ten feet, producing long floating branches during the summer. Another conspicuous alga of the *Calliarthron* zone is *Desmarestia herbacea*. Numerous red algae characteristic of the low intertidal zone may extend into the subtidal region. The distribution and zonation of these red algae are dependent upon complex factors not considered here, major attention having been directed to the deeper zones. The over-all color of the *Calliarthron* zone is red, due to the alga itself and to *Lithothamnion lamellatum* and *L. californica,* which cover most of the remaining rock surfaces.

Few invertebrates are conspicuous in this zone. Large sessile animals seem unable to compete for space with the dense growth of *Calliarthron*. However, some forms characteristic of the intertidal zone of more protected environments replace *Calliarthron* in crevices. Only a few abundant animals in this zone were considered. The large gill fans of the sabellid worm, *Eudistylia polymorpha,* are conspicuous projecting from crevices. Large leaf-like colonies of the bryozoan *Hippodoplosia insculpta* are also able to gain a foothold among the *Calliarthron*.

THE PTERYGOPHORA ZONE

As the diver descends a typical vertical rock wall, the growth of *Calliarthron* becomes thinner and widely scattered (Fig. 2). Large *Laminaria setchellii* are spaced closer than a foot apart and grow out from the walls at a 45° angle. At a depth of 15 feet a large *Laminaria* may have a stipe five feet in length and a blade area of six square feet. With increasing depth, a profusion of sponges and tunicates appears on the walls. An orange sponge, *Tethya aurantia californica,* several inches in diameter, is conspicuous. On overhung walls, a red alga, *Fryeela gardneri,* produces an iridescent horizontal blade. Colonies of the anemone *Corynactis californica* are plentiful, and the solitary coral *Balanophyllia elegans* is abundant on all rock surfaces. In caves and overhung areas, a dark blue sponge, *Hymenamphiastra cyanocrypta,* covers large areas; a red anemone, *Tealia lofotensis,* is also found here. Sessile animals are most abundant on vertical rock surfaces below 20 feet where competition for space with *Calliarthron* is less extreme. Organisms common on vertical rock walls are listed in Table I.

Large boulders and rock ledges comprise the bottom at depths of 30 to 50 feet. Granitic gravel and shell fragments fill depressions among rocks. Aggregations of the polychaete worm *Diopatra ornata*, with tubes composed of broken shell, are conspicuous at the surface of the gravel. Algal detritus accumulates in thick masses in deep pockets among the bottom reefs.

The bottom rocks support a forest of the laminarian kelp *Pterygophora cali-*

fornica. A typical *Pterygophora* plant has the general appearance of a palm tree, with a flattened erect stipe an inch in width and three to five feet long, and about 20 blades having a combined surface area(on one side) of approximately 18 square feet. The stipes are often closer than a foot apart. A rather heavy subsurface canopy is therefore produced, and the diver, swimming above it, will see little of the life beneath this canopy. *Pterygophora* is a perennial plant that lives for several years. The holdfast is nearly as large as that of *Nereocystis.* Unlike the *Nereocystis* stipe, that of *Pterygophora* is woody and slow to decompose. Numbers of detached stipes accumulate in the pockets between rocks.

TABLE I

Organisms common on vertical rock surfaces at depths of 20 feet and below

Algae
Laminaria setchelii
Calliarthron cheilosporioides
Fryeella gardneri

Porifera
Leucoselenia eleanor
Leuconia heathi
Rhabdodermella nuttingi
Tethya aurantia californica
Hymenamphiastra cyanocrypta

Hydrozoa
Abietinaria abietina
Aglaophenia latirostris

Anthozoa
Balanophyllia elegans
Corynactis californica
Anthopleura xanthogrammica
Tealia lofotensis

Bryozoa
Diaperoecia californica
Hippodiplosia insculpta
Phidolopora pacifica

Polychaeta
Eudistylia polymorpha
Serpula vermicularis

Polychaeta (cont.)
Pista elongata

Cirripedia
Tetraclita squamosa elegans

Brachyura
Loxorhynchus crispatus

Amphineura
Tonicella lineata

Gastropoda
Diodora aspera
Calliostoma ligatum
Ceratostoma foliatum
Ocenebra lurida
Fusinus luteopictus

Pelecypoda
Hinnites multirugosus

Asteroidea
Henricia leviuscula annectens

Ascidiacea
Amaroucium solidum
Clavelina huntsmani
Distaplia occidentalis
Eudistoma sp.
Polyclinum planum

The streamer kelp *Costaria costata* is found with *Pterygophora. Costaria* has an undivided blade over a foot in width and about five feet long that trails along the bottom under the *Pterygophora* canopy. The large blades reach full size in June. Another major alga of the *Pterygophora* zone is *Desmarestia munda,* with branching blades several feet in length. Other common algae include *Dictyota cribosa, Plocamium pacificum* and *Polysiphonia paniculata,* each of which grows in conspicuous tufts on tops of boulders not heavily shaded by *Pterygophora.* During the winter and early spring months the absence of *Nereocystis, Costaria, Desmarestia, Cystoseira* and *Egregia* is very noticeable. The diver is no longer in a jungle.

Calliarthron, Laminaria, Dictyoneurum, and *Pterygophora* remain. The conspicuous smaller algae, such as *Dictyota, Plocamium* and *Fryeella,* are likewise reduced during winter months.

One of the more conspicuous invertebrates in the surge channels of the *Pterygophora* zone is the giant green anemone *Anthopleura xanthogrammica;* individuals are often spaced a few feet apart. *Patiria miniata* is the most common starfish and the many-rayed star *Pyncnopodia helianthoides* is often encountered. Mollusks are conspicuous. The large gumboot chiton *Cryptochiton stelleri* is common and *Tonicella lineata* is an abundant chiton seen on all rock surfaces. Aspidobranch gastropods are abundant as species and individuals; an ideal environment is provided for their grazing manner of feeding. *Tegula brunnea* is a common gastropod associated with brown algae; the small *Homalopoma carpenteri* is another abundant gastropod.

TABLE II

Organisms common on level rock surfaces under the Ptergophora canopy

Algae

Pterygophora californica
Costaria costata
Desmarestia munda
Dictyota cribosa
Plocamium pacificum
Polysiphonia paniculata

Porifera

Tedania topsenti

Bryozoa

Crisia maxima

Sipunculoidea

Phascolosoma agassizi

Anomura

Pagurus hemphillii

Anomura (cont.)

Paguristes ulreyi

Amphineura

Cryptochiton stelleri

Gastropoda

Acmaea mitra
Acmaea ochracea
Astraea gibberosa
Homalopoma carpenteri
Petaloconchus montereyensis

Asteroidea

Patiria miniata
Pyncnopodia helianthoides

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The fauna under the *Pterygophora* canopy and on the sides of boulders is not as rich as that found on vertical walls. However, in sites which are well-protected by dense growth of *Pterygophora,* the rock may be encrusted with organisms up to an inch in thickness. These mats contain loose layers of *Lithothamnion,* colonies of the orange sponge *Tedania topsenti,* the feathery bryozoan *Crisia maxima,* small sabellid worms, the sipunculid *Phascolosoma agassizi,* the sessile gastropod *Petaloconchus montereyensis,* and numerous other small organisms. On sloping sides of boulders and other areas not protected by a heavy *Pterygophora* canopy, this mat of organisms is not present and is replaced by red corallines such as *Lithothamnion, Bossiella,* and *Corallina.* Organisms common on level rock surfaces are listed in Table II.

Many motile invertebrates and encrusting forms are found under loose rock in the channels between reefs. Some of the common animals found under rock or on the under surfaces of rock are listed in Table III.

Still another association occurs on the stipes and fronds of *Laminaria* and *Pterygophora,* which provide a substrate for gastropods, bryozoans and hydroids. Associated animals are listed in Table IV.

SEA OTTER PREDATION

One large invertebrate that might normally be expected was not found in the areas I studied. Living *Strongylocentrotus franciscanus,* the large sea urchin, was totally absent, although spines and test fragments were present in gravel samples. This appears to be the result of predation by the California sea otter, *Enhydra lutris nereis,* maximum populations of which are now found between the Monterey Peninsula and San Simeon, California (Boolootian, 1961). I have occasionally observed them in my diving area. Dr. Richard Boolootian spent eight to ten hours daily during May and June, 1956, observing a herd of 50 animals through a telephoto lens from shore at Rocky Point, two miles to the south of the present area. Fifty sea otters were observed eating 5280 *S. franciscanus,* 301 *Mytilus californianus,* and 380 *Haliotis rufescens* (Boolootian, personal communication). They were found to entirely clean out one urchin bed and move on to another. Their effect upon abalone populations is not fully known. A few large *Haliotis* were seen in crevices on the rock walls. The smaller *Haliotis wallalensis* is common under the bottom rocks, although adults of this species may also suffer predation.

TABLE III

Organisms common on under surfaces of loose bottom rocks

Brachyura	*Mimulus foliatus*
	Pugettia richii
	Scyra acutifrons
Amphineura	*Ischnochiton radians*
	Lepidozona mertensii
Gastropoda	*Haliotis wallalensis*
Ophiuroidea	*Ophioplocus esmarki*
	Ophiothrix spiculata

TABLE IV

Animals associated with stipes and fronds of Laminaria and Pterygophora

Hydrozoa	*Plumularia lagenifera*
Bryozoa	*Membranipora membranacea*
Gastropoda	*Acmaea instabilis*
	Tegula brunnea
	Tegula montereyi

The fact that as many as 5280 urchins were taken from a *Nereocystis* community similar to the one I have observed indicates that these urchins would be dominant members of the community in the absence of the otters. Populations of large subtidal urchins are found in many temperate areas of the world and their grazing effects are considerable. Forster (1959) calculated a density of 868 *Echinus esculentus* per acre near Plymouth, England, and determined that their rate of browsing on algae and sessile animals would sweep clear at least one-third of the rock surface in the course of a year. The effect of *S. franciscanus* has been noted in *Macrocystis* beds in Baja California. Dawson *et al.* (1960, p. 12) reported: "A grounded tanker released crude oil into a small bay on the Baja California coast, killing nearly all of the animals, including the major herbivores such as the sea urchins and abalone. The great increase and luxuriance of plant growth that followed upon the removal of grazing pressure dramatically demonstrated the important part played by these animals in the regulation of the plant association." Grazing effects of *S. franciscanus* have been noted also by McFarland and Prescott (1959), and Limbaugh (1955). Apparently the otters are permitting luxuriant development of the *Nereocystis-Pterygophora* association by their predation upon urchins and, to a lesser extent, abalones. The otters do not range into Monterey Bay. The subtidal rocks in Monterey Bay at Mussel Point at depths of 10 to 20 feet are covered with urchins and abalones spaced only a few feet apart. Although conditions are too calm for good development of *Nereocystis* and *Pterygophora*, all other large algae are heavily grazed upon, and the general appearance of these rocks is barren.

Because of the value of their fur, the sea otters were brought to the brink of extinction by the early part of this century, but under heavy protection they have recently increased in numbers. It is altogether likely that during the period in which they were scarce the urchins were found abundantly in the *Nereocystis-Pterygophora* association and kept this community at a minimum level of development as a result of grazing.

Discussion

In the relatively uniform environment investigated, I have recognized two zones based upon the dominant species of algae in each zone, the immediate subtidal or *Calliarthron* zone, and the deeper *Pterygophora* zone. No well-defined separation exists between the zones I have designated. The usual transition on vertical walls involves a thinning of the *Calliarthron* with increasing depth; replacement is accomplished with large *Laminaria, Pterygophora,* and *Costaria,* and increasing numbers of sessile animals. Thickest growth of *Calliarthron* occurs on projecting walls exposed to continual action of surge below the surf areas. Calmer water and some shade are associated with the position of *Pterygophora*. Fully developed plants of the latter alga will not be found shallower than 20 feet.

Kelp zones characteristically extend to depths of 60 to 70 feet; below these depths algae are usually limited to inconspicuous red forms. Forster (1954) examined the transition between the laminarian and sub-laminarian zones near Plymouth, England. Limbaugh and Shepard (1957) described some of the fauna of this deeper zone in the Scripps and La Jolla Submarine Canyons of southern California. The transition could not be observed on the coastal area under consideration because sand replaces the granite boulders at depths of 50 to 60 feet. However, underwater observations at depths of 70 to 100 feet at the head of the Carmel Submarine Canyon, six miles to the north, have confirmed the appearance of many faunal elements including a gorgonian, several anemones, sponges and red algae. A faunal survey of the walls of this canyon is now in progress.

Andrews (1925) studied the fauna associated with *Nereocystis* in Puget Sound and found that the canopy supported only the minute gastropod *Lacuna* and a few crustaceans. I have noticed no particular species associated with the *Nereocystis* canopy in the present areas. This may be due to the annual life-cycle of the plant and its slimy surface. In contrast, the stipes and fronds of *Macrocystis* support bryozoans, hydroids, gastropods, and many other forms (Limbaugh, 1955). Five top shells are found on *Macrocystis* stands in Carmel Bay: *Tegula brunnea, T. montereyi, T. pulligo, Calliostoma annulatum* and *C. canaliculatum*. The three species of *Tegula* reach comparable sizes on *Laminaria* and *Pterygophora* but the two *Calliostoma* seem to achieve maximum development only on *Macrocystis*.

Holdfasts of kelps likewise create important microhabitats for many small animals. Andrews (1945) examined holdfasts of *Macrocystis* from Carmel and Monterey bays and tabulated seasonal changes in the composition of the holdfast fauna, which included many small species not considered in the present report. He found that the majority of the animals were immature stages of species not restricted to holdfasts, the kelp holdfast providing protection during the early period of growth. He had found (Andrews, 1925) that the fauna of holdfasts of *Nereocystis* in Puget Sound yielded some 40 species of gastropods, polychaetes, brittle stars and amphipods. The *Pterygophora* holdfast is as large as that of *Nereocystis* and probably supports as many animals.

A considerable amount of light is removed by the *Nereocystis* canopy during the summer. Most of the light which penetrates through this canopy is then absorbed by the heavy subsurface canopy of *Pterygophora* and *Costaria*. Measurements taken under the *Pterygophora* canopy with a photoelectric light meter in a glass jar showed a loss of over 95% of the light available immediately below the surface. Loss of this amount of light characterizes kelp forests of temperate coasts. Kitching (1941) found that laminarian kelps at Carsaig Island, Scotland, cut off 99% of the available light at depths of one to six meters, the illumination changing very little over this depth range. Similar light penetration measurements have also been made within southern California *Marcocystis* beds by McFarland and Prescott (1959).

Kelp communities are highly productive. McFarland and Prescott (1959) measured wet standing crop, chlorophyll content, and *in situ* metabolism of a giant kelp community in southern California and found that a *Macrocystis* kelp bed produces yields comparable to other highly productive communities such as coral reefs and marine grass flats. The *Nereocystis-Pterygophora* community probably has a similar productive capacity, judging from the amount of light absorbed.

The *Macrocystis* beds of southern California have become relatively well-known through recent publications (Limbaugh, 1955; Aleem, 1956; McFarland and Prescott, 1959; Dawson, Neushul and Wildman, 1960). The *Nereocystis-Pterygophora* beds differ chiefly in their inshore position and in the annual life-cycle of *Nereocystis*. During the winter they are more comparable to laminarian kelp beds studied in the European north Atlantic (Gislén, 1930; Kitching, Macon and Gilson, 1934; Kitching, 1941; Drach, 1949; Forster, 1958; Kain, 1960). The north Atlantic fauna reported by Forster is similar in many ways to that in the present study. He found a similar rich development of *Corynactis,* sponges, bryozoans, and tunicates. Many genera are represented in both localities. A major difference is the scarcity of gastropods, other than nudibranchs in the sublittoral zones near Plymouth. In the *Nereocystis-Pterygophora* beds they are well represented in species and individuals. For a comparison of common sublittoral forms on the West Coast, descriptions of areas with faunal listings are given by Limbaugh (1955) and Pequegnat (1961) for southern California, and by Shelford *et al.* (1935) for Puget Sound in Washington.

List of Species

An endeavor has been made in the listings that follow to include all common invertebrates and algae that may be taken on rock walls and level bottoms below 20 feet, down to a depth of 50 feet. Such groups as the small crustaceans, free-living polychaete worms and others have been left out entirely. Mollusks and algae have been treated in greatest detail.

The following have assisted me with identifications: Dr. Isabella Abbott and Dr. George J. Hollenberg (algae), Dr. E. Yale Dawson (coralline algae), Dr. Cadet Hand (hydroids), Dr. John D. Soule (bryozoans), Dr. Cyril Berkeley (tubiculous polychaetes), Mr. Victor Zullo (barnacles), Dr. Rolf Bolin (decapod crustaceans), Dr. A. Myra Keen and Mr. Allyn G. Smith (mollusks), and Dr. Donald P. Abbott (tunicates). Many of the invertebrates mentioned are included in the keys given by Light *et al.* (1957). Ricketts' and Calvin's *Between Pacific Tides* is also a valuable reference for this fauna. Both books contain bibliographies that include the more important systematic papers dealing with Pacific Coast fauna.

Species that are limited to subtidal zones or are rare intertidally in the Monterey area are indicated by an asterisk. Species marked with a double asterisk are rare at depths to 50 feet but are common elsewhere in the Monterey area at depths below 70 feet, according to my underwater observations. Organisms listed as abundant may be collected in large numbers during a single dive. Limited numbers of those listed as common may usually be collected during a dive, while "scarce" species may not always be found during a dive, even after intensive search.

MARINE ALGAE

Chlorophyta

Enteromorpha sp. Scarce on unshaded horizontal surfaces.
Cladophora microcladioides Collins. On vertical rock walls.
Spongomorpha coalita (Rupr.) Collins. On walls not heavily shaded.
Derbesia marina (Lyng.) Solier. The *Halicystis*-phase is common on *Lithothamnion* on vertical walls.

Phaeophyta

Ectocarpus acutus S. & G. Abundant epiphyte on *Desmarestia munda* and *D. herbacea*.
Ectocarpus confervoides var. *pygmaeus* (Aresch.) Kjellm. Epiphytic on floats of *Nereocystis*.
Sphacelaria didichotoma Saund. Recorded on *Pterygophora* holdfasts.
**Dictyota cribosa* S. & G. Common on tops of boulders in early summer.
Desmarestia herbacea (Turn.) Lamour. Common in *Calliarthron* zone in early summer.

Laminaria setchellii (Eaton) Silva. (Syn. *L. andersonii.*) Conspicuous on vertical walls throughout the year.

Costaria costata (Turn.) Sand. Common with *Pterygophora* during the summer and fall.

Dictyoneurum californicum Rupr. Common in *Calliarthron* zone throughout the year.

Nereocystis luetkeana (Mert.) P. & R. Holdfasts on tops of boulders during the summer and fall.

**Macrocystis pyrifera* (L.) C. A. Ag. Scattered plants throughout the year.

**Pterygophora californica* Rupr. Abundant below 20 feet throughout the year.

Egregia menziesii (Turn.) Aresch. Holdfasts at mean low tide and tops of boulders in shallow water during the summer and fall.

Cystoseira osmundacea (Menzies) C.A.Ag. Best developed in *Calliarthron* zone during the summer, non-fruiting plants at greater depths.

Rhodophyta

Pikea californica Harvey. Scarce under *Pterygophora* canopy.

Peyssonnelia pacifica Kylin. Common encrusting *Tegula* shells.

Lithothamnion californica Foslie. Covers most rock surfaces free of sessile animals.

Lithothamnion conchatum Setch. & Fosl. Epiphytic on *Calliarthron.*

Lithothamnion lamellatum Setch. & Fosl. Forms large crustose sheets in *Calliarthron* zone.

Lithophyllum lichenare L. R. Mason. Common in *Pterygophora* zone.

Bossiella californica (Dcne.) Silva. Common under *Pterygophora* canopy.

Corallina chilensis Dcne. Common on horizontal surfaces under *Pterygophora* canopy.

Calliarthron cheilosporioides Manza. Covers the rock surface in the immediate subtidal zone. Scattered plants at greater depths.

Plocamium pacificum Kylin. Forms conspicuous tufts on tops of boulders not heavily shaded.

Fauchea media Kylin. Scarce on walls at 20 feet.

**Fryeella gardneri* (Setch.) Kylin. Common on shaded vertical and overhung walls.

Rhodomenia californica Kylin. Common on holdfasts and rock surfaces under the *Pterygophora* canopy.

**Antithamnion defectum* Kylin. Scarce on rock walls.

Griffithsia pacifica Kylin. Scarce on rock walls.

Microcladia coulteri Harv. An abundant epiphyte on holdfasts of *Pterygophora.*

Delesseria decipiens J. G. Ag. Scarce on rock walls at 20 feet.

Polyneura latissima (Harv.) Kylin. Small specimens at 20 feet.

Botryoglossum farlowianum (J. G. Ag.) De Toni. Scarce under the *Pterygophora* canopy.

**Dasyopsis densa* G. Sm. Scarce on rock walls.

Polysiphonia pacifica var. *delicatula* Holl. Abundant on exposed bottom rocks in April, 1961.

Polysiphonia paniculata Mont. (Syn. *P. californica*) In tufts on tops of boulders.

Pterosiphonia baileyi (Harv.) Falken. On rock walls.

Pterosiphonia dendroidea (Mont.) Falken. Common on holdfasts and *Lithothamnion.*

**Pterosiphonia gracilis* Kylin. Recorded on the surface of a colonial tunicate.

Herposiphonia rigida Gardn. Common on *Lithothamnion* and holdfasts.

Amplisiphonia pacifica Holl. On *Pterygophora* holdfasts.

PORIFERA

Calcarea

Leucoselenia eleanor Urban. Finely branched grey masses common on rock walls.

**Sycon coronatum* (Ellis and Solander). A small solitary urn-shaped form scarce on rock walls.

Leuconia heathi (Urban). A sharp-spined globular form common in crevices on rock walls.

Rhabdodermella nuttingi Urban. An urn-shaped form common on walls.

Demospongiae

**Tethya aurantia californica* de Laub. A globular orange form abundant in crevices on walls.

***Polymastia pachymastia* de Laub. Large yellow colonies scarce on flat surfaces and walls.

**Prianos problematicus* de Laub. A soft drab-colored amorphous sponge on walls.

**Tedania topsenti* de Laub. A stiff orange form common on walls and flat surfaces under *Pterygophora.*

Hymenamphiastra cyanocrypta de Laub. An encrusting purple form on heavily shaded walls.

**Gellius edaphius* de Laub. A stiff drab form scarce on walls.

Aplysilla polygraphis de Laub. Large purple encrusting colonies scarce on walls.

COELENTERATA

Hydrozoa

Eudendrium californicum Torrey. Large branching colonies scarce on walls.

Abietinaria abietina (Linnaeus). Large dark-colored plumes common on walls.

Abietinaria greenei (Murray). A thickly growing tufted form common on walls.

Aglaophenia cf. *A. latirostris* Nutting. A plumed form common on walls.

Sertularella sp. A large plumed form common on walls.

Scyphozoa

Haliclystus sp. A small sessile jellyfish scarce on rock walls.

Anthozoa

Balanophyllia elegans Verrill. An abundant orange-colored coral on vertical rock surfaces.

***Paracyathus stearnsii* Verrill. A large solitary coral scarce at 40 feet.

Anthopleura xanthogrammica (Brandt). The giant green anemone, conspicuous in crevices and deep surge channels.

Epiactis prolifica Verrill. A small anemone common on walls.

Tealia coriacea (Cuvier). A red anemone common on walls and in crevices.

Tealia crassicornis (Müller). A mottled green and red anemone on walls and under *Pterygophora*.

**Tealia lofotensis* (Danielssen). A large red anemone common on walls.

?*Harenactis* sp. A small burrowing anemone common on gravel surfaces.

Corynactis californica Carlgren. A small club-tentacled anemone forming colonies on shaded walls.

BRYOZOA

Ctenostomata

Flustrella corniculata (Smitt). A large encrusting form common on stems of *Calliarthron.*

Cyclostomata

**Crisia maxima* Robertson. Grows erect under the *Pterygophora* canopy.

**Diaperoecia californica* (Orbigny). Large branching colonies common on vertical walls.

**Heteropora alaskensis* (Borg). Small branching colonies scarce on walls.

**Lichenopora* cf. *L. novae-zelandiae*. Large purple encrustations scarce on walls.

Cheilostomata

Bugula californica Robertson. Finely branched colonies scarce on walls.

Dendrobeania longispinosa (Robertson). An encrusting form often living on shells of *Diodora aspera.*

Hippodoplasia insculpta (Hincks). Erect leaf-like colonies abundant in *Calliarthron* zone, less common in deeper water.

Lyrula hippocrepis (Hincks). An encrusting form commonly found on shells of *Ceratostoma foliatum.*

Membranipora membranacea (Linnaeus). An encrusting form found abundantly on fronds of *Laminaria* and *Pterygophora.*

Parasmittina collifera (Robertson). An encrusting form on shells of *Diodora.*

Phidolopora pacifica (Robertson). A lace-like erect form common on walls.

Rynchozoon tumulosum (Hincks). An encrusting form on shells of *Diodora.*

Tricellaria praescuta Osburn. A finely-branched form commonly attached to sponges and algae.

POLYCHAETA

Dodecaceria concharum Oersted. An abundant cirratulid which bores in *Lithothamnion.*

Sabellaria cementarium Moore. A sabellariid forming tubes attached to undersides of rocks.

Eudistylia polymorpha (Johnson). A large sabellid, abundant in crevices, especially in *Calliarthron* zone.

Serpula vermicularis Linnaeus. Calcareous tubes common in crevices.

**Spirorbis ambilateralis* Pixell. Common on *Diodora* shells and rocks. Previously known only from Vancouver Island.

Spirorbis spirillum (Linnaeus). Abundant on algae and rocks.

Pista elongata Moore. A terebellid producing tubes with sponge-like openings, common in crevices on rock walls.

SIPUNCULOIDEA

Phascolosoma agassizii Keferstein. Common nestling in holdfasts and sponges.

ARTHROPODA

Cirripedia

Balanus crenatus Brugière. A small form found on *Diodora aspera* shells and rocks.

Balanus nubilus Darwin. A large form recorded on *Diodora*.

Balanus tintinabulum (Linnaeus). A reddish form recorded on *Diodora*.

Tetraclita squamosa elegans Darwin. A large form common on rock walls.

Brachyura

Cancer antennarius Stimpson. A large form on gravel bottoms.

Cancer jordani Rathbun. Small specimens scarce on gravel bottoms.

Cancer productus Randall. Juveniles common on gravel.

Lophopanopeus heathii Rathbun. A small form common on gravel.

Lophopanopeus leucomanus (Lockington). Common on gravel.

**Loxorhynchus crispatus* Stimpson. The masking crab; large individuals cling to vertical walls.

Mimulus foliatus Stimpson. A small red form common under rocks and on gravel.

Pugettia producta (Randall). The kelp crab, on walls and large brown algae.

Pugettia richii Dana. A small form in crevices and under rocks.

Scyra acutifrons Dana. A small form common in crevices and under rocks.

Anomura

Cryptolithodes sitchensis Brandt. A scarce rock crab, found clinging to walls.

Haplogaster cavicauda Stimpson. Scarce in crevices and under rocks.

***Phyllolithodes papillosus* Brandt. An ornate rock crab, scarce on rock walls.

Pagurus granosimanus (Stimpson). A small hermit crab on gravel bottoms.

Pagurus hemphillii (Benedict). A hermit crab using *Tegula* shells, common on gravel and rock bottoms.

**Paguristes ulreyi* Schmidt. A large hermit crab using *Astraea* and *Ceratostoma* shells, common on gravel and rock bottoms.

MOLLUSCA

Amphineura

Tonicella lineata (Wood). The lined chiton, abundant on all rock surfaces.
Basiliochiton heathii Pilsbry. Uncommon on under surfaces of rocks.
Mopalia sp. Uncommon on bottom rocks.
Mopalia lignosa (Gould). Common on bottom rocks.
Placiphorella velata Dall. In crevices and on under sides of rocks.
Cryptochiton stelleri (Middendorff). The gum boot chiton, common on rock surfaces.
Ischnochiton radians Carpenter. Common on under surfaces of rocks.
Ischnochiton regularis (Carpenter). Scarce on under surfaces of rocks.
Lepidozona mertensii (Middendorff). Common on under surfaces of rocks.
Stenoplax fallax Pilsbry. A red form scarce on under surfaces of rocks.

Gastropoda

Acmaea instabilis (Gould). An abundant limpet on *Laminaria* and *Pterygophora* stipes.
Acmaea mitra Eschscholtz. A common white limpet known to feed on *Lithothamnion.*
Acmaea ochracea Dall. Common on and under bottom rocks.
Acmaea pelta Eschscholtz. Abundant in the intertidal zone, a few individuals occur in deeper water.
Acmaea rosacea Carpenter. A small scarce form on rocks.
Acmaea triangularis (Carpenter). A small limpet on *Calliarthron,* rocks and shells.
Haliotis rufescens Swainson. The red abalone, uncommon in protected crevices.
**Haliotis wallalensis* Stearns. Common on under surfaces of rocks.
Diodora aspera (Eschscholtz). Large specimens common on rock walls.
**Diodora murina* (Dall). Scarce on under surface of rocks.
Megatebennus bimaculatus (Dall). Scarce on bottom rocks.
**Calliostoma annulatum* (Humphrey). Juveniles common on rock walls.
Calliostoma canaliculatum Dillwyn. Juveniles on bottom rocks.
Calliostoma ligatum (Gould). Abundant on rock walls.
Calliostoma splendens Carpenter. A small form scarce on bottom rocks.
**Margarites parcipictus* (Carpenter). A minute form abundant under rocks.
Margarites salmoneus (Carpenter). Scarce on under surfaces of rocks.
Tegula brunnea (Philippi). Juveniles on rock, adults on *Laminaria* and *Pterygophora.*
**Tegula montereyi* (Kiener). Common on *Macrocystis, Pterygophora* and *Laminaria.*
Tegula pulligo (Gmelin). Juveniles on rocks, adults on *Laminaria* and *Pterygophora.*
**Liotia acuticostata* (Carpenter). Shells found in coarse gravel.
**Astraea gibberosa* (Dillwyn). A large form on rock surfaces under *Pterygophora.*
Homalopoma carpenteri Pilsbry. An abundant small form on and under rocks.
Tricolia pulloides (Carpenter). A small form in gravel and under rocks.

Tricolia rubrilineata (Strong). A minute form common on and under bottom rocks.

**Balcis thersites* (Carpenter). Scarce under bottom rock.

**Epitonium indianorum* (Carpenter). Scarce in gravel.

Alvania purpurea Dall. Shells common in gravel.

Barleeia oldroydi Bartsch. A minute form, seasonally abundant under rocks and in gravel.

Diala acuta Carpenter. Common in gravel and under bottom rocks.

Petaloconchus montereyensis (Dall). Colonies on upper surfaces of rocks under *Pterygophora*.

**Petaloconchus* sp. A dark colored form found especially in *Diodora* shells.

Serpulorbis sp. Attached to vertical rock surfaces.

Bittium attenuatum Carpenter. Scarce in gravel and under bottom rocks.

Bittium interfossa Carpenter. Shells found in coarse gravel.

Cerithiopsis diegensis Bartsch. Scarce under bottom rocks.

***Seila montereyensis* Bartsch. Scarce under bottom rocks.

Crepidula adunca Sowerby. Common attached to all large gastropod shells.

Crepidula perforans (Valenciennes). On rock or in apertures of shells occupied by *Pagurus*.

Crepipatella lingulata (Gould). Common on surfaces of small rocks and shells.

Hipponix tumens Carpenter. Shells found in gravel.

Lamellaria rhombica Dall. Scarce on bottom rocks and on gravel.

Velutina laevigata (Müller). Scarce on bottom rocks and on gravel.

Trivia californiana Gray. Shells found in coarse gravel.

***Murex carpenteri* (Dall). Scarce on bottom rocks.

Ceratostoma foliatum (Gmelin). A large murex, common on bottom rocks and in crevices on walls.

***Ocenebra beta* (Dall). Scarce on bottom rocks.

Ocenebra interfossa Carpenter. On bottom rocks.

Ocenebra lurida (Middendorf). Common on all rock surfaces.

**Amphissa columbiana* Dall. Under bottom rocks.

Amphissa versicolor Dall. Common on and under bottom rocks.

Mitrella carinata (Hinds). Common on rock surfaces and algae.

Mitrella tuberosa (Carpenter). Under bottom rocks and on gravel.

Nassarius mendicus (Gould). Scarce on bottom rocks.

Fusinus luteopictus (Dall). Common on rock surfaces.

**Fusinus monksae* Dall. Scarce on rock surfaces under *Pterygophora* canopy.

Cypraeolina pyriformis (Carpenter). Common under rocks and on gravel.

Mangelia interlirata Stearns. Scarce on bottom rocks.

Turbonilla (*Pyrgiscus*) sp. Scarce on gravel surfaces.

Turbonilla (*Strioturbonilla*) sp. Scarce on gravel surfaces.

Williamia vernalis (Dall). A small pulmonate limpet scarce on rock surfaces.

Nudibranchia

Triopha carpenteri (Stearns). Large specimens of this red and white form common on rock walls.

Cadlina marginata MacFarland. A white form common on rock walls.

Anisodoris nobilis (MacFarland). On rock walls.
Dialula sandiegensis (Cooper). Scarce on rock walls.
Glossodoris macfarlandi (Cockerell). Scarce on rock walls. Known range extended north from San Pedro, California.
Dendrodoris fulva MacFarland. A yellow form common on walls and bottom rocks.
Dendronotus sp. Scarce on rock walls.
Aeolidia papillosa (Linnaeus). Scarce on rock walls.
Hermissenda crassicornis (Eschscholtz). Common on rock walls and bottom rocks.

Pelecypoda

***Chlamys hastatus* (Sowerby). A scarce free-swimming scallop.
Hinnites multirugosus (Gale). The rock scallop, cemented to rock walls.
***Lima hemphilli* Hertlein and Strong. A scarce free-swimming form.
Pododesmus cepio (Gray) Small specimens common on shells and rocks.
Mytilus californianus Conrad. Juveniles on upper surfaces of boulders, apparently torn loose from intertidal zone by storms.
**Modiolus fornicatus* (Carpenter). Nestling in gravel and tunicates.
Mytilimeria nuttallii Conrad. Common in cavities surrounded by compound ascidians.
Milneria kelseyi (Dall). Valves scarce in gravel.
Chama pellucida Broderip. A fixed rock clam in crevices and under sides of rocks.
Epilucina californica (Conrad). Scarce in bottom gravel.
Kellia laperousii (Deshayes). Small specimens commonly nestling in holdfasts and crevices.
Gari californica (Conrad). Scarce in bottom sand and gravel.
Schizothaerus nuttallii Conrad. Scarce in sandy areas away from rocks.
Hiatella arctica (Linnaeus). Common nestling in holdfasts and crevices.

Cephalopoda

Octopus appollyon Berry. Small specimens scarce in rock crevices.

ECHINODERMATA

Asteroidea

**Dermasterias imbricata* (Grube). The leather star, scarce on rocks and walls.
**Evasterias troschelii* (Stimpson). Scarce on walls.
Henricia leviuscula (Stimpson). A small form on rock surfaces.
**Henricia leviuscula* var. *annectens* Fisher. A larger form common on rock walls.
Leptasterias aequalis (Stimpson). A common small form on and under rocks.
Patiria miniata (Brandt). The bat star, conspicuous on bottom rocks.
**Pisaster giganteus* (Stimpson). Scarce on rocks and walls.
***Poraniopsis inflata* Fisher. Scarce on rock walls.
**Pycnopodia helianthoides* (Brandt). The large many-rayed star, common on bottom rocks.
***Stylasterias forreri* (de Loriol). Scarce at depths of 40 feet.

Ophiuroidea

Amphiodia occidentalis (Lyman). Buried in bottom sand and gravel.
**Amphipholis pugetana* (Lyman). Common on bottom sand and gravel.
Ophioplocus esmarki Lyman. A large form common in crevices and under rocks.
Ophiopholis aculeata forma *kennerlyi* (Lyman). Scarce in gravel.
**Ophiopteris papillosa* (Lyman). A large form scarce in crevices.
Ophiothrix spiculata LeConte. Abundant in crevices and under rocks.

Echinoidea

Strongylocentrotus purpuratus (Stimpson). The small purple urchin; forms extensive beds in the low intertidal zone; small green juveniles common in deeper water.
Strongylocentrotus franciscanus (Agassiz). The large purple urchin, living juveniles scarce; test fragments of adults only.

Holothuroidea

Eupentacta quinquesemita (Selenka). A white form scarce on rock walls.
***Psolus chitonoides* Clark. A flat-sided red form scarce on walls.
***Cucumaria miniata* Brandt. A large drab-colored form scarce in crevices.
**Cucumaria piperata* (Stimpson). A small spotted form uncommon in crevices.

ASCIDIACEA

Aplousobranchia

Amaroucium solidum Ritter and Forsyth. Large red colonies common on walls.
Clavelina huntsmani Van Name. A pink form common on walls.
Cystodytes sp. A common translucent form on walls.
Didemnum carnulentum Ritter and Forsyth. Small white colonies common on walls.
Distaplia occidentalis Bancroft. A variable-colored pedunculate form common on walls.
Distaplia sp. A pale pink form uncommon on overhung surfaces.
Eudistoma psammion Ritter and Forsyth. A dark-brown form common on walls.
Eudistoma sp. An abundant grey form on walls.
Polyclinum planum (Ritter and Forsyth). Large pedunculate colonies common on walls.
Pycnoclavella stanleyi Berrill and Abbott. A small orange form common on walls.
Trididemnum opacum (Ritter). Large white colonies encrusting walls and under surfaces of rocks.

Phlebobranchia

Chelysoma productum Stimpson. Scarce on walls and under surfaces of rocks.
Perophora annectens Ritter. A small green form common on walls and holdfasts.

Stolidobranchia

**Boltenia villosa* (Stimpson). A spiny solitary form common in crevices on walls.

**Cnemidocarpa finmarkiensis* (Kiaer). A smooth pink form, scarce on undersurfaces of bottom rocks.

**Pyura haustor* (Stimpson). A large wrinkled solitary form, common in crevices.

Metandrocarpa taylori Huntsman. A small red form common on walls.

**Styela gibbsii* (Stimpson). Common in crevices and on sides of rocks.

Styela montereyensis (Dall). A stalked form scarce on walls.

I am indebted to Dr. Donald P. Abbott, Dr. Rolf Bolin and Dr. Arthur C. Giese, of Stanford, for helpful suggestions during the course of this survey. Specialists who have contributed identifications are mentioned in the listings, and I acknowledge my indebtedness to them. This survey could not have been pursued without the help of those who have assisted me in the field and have dived with me, particularly Joe Brumbaugh, David Caldwell, William Coit, and Michael Ghiselin.

SUMMARY

1. Aqualung dives along the open coast south of Carmel, California, have been made over a period of two years.

2. The predominant kelp on the open coast in the Monterey area is *Nereocystis luetkeana;* the giant kelp *Macrocystis pyrifera* is scarce. *Nereocystis* is an annual plant producing a heavy subsurface canopy in the summer months.

3. Inshore water is deep; the granite walls drop to a depth of 10 to 30 feet. A dense growth of the branching red coralline alga *Calliarthron cheilosporioides* carpets the immediate subtidal rocks to a depth of 10 feet, to the exclusion of most sessile animals.

4. Below this zone large *Laminaria setchellii* replace *Calliarthron* and a profusion of sponges and tunicates occurs on the walls. The rock bottom at depths of 20 to 50 feet supports a forest of the perennial kelp *Pterygophora californica* which produces a subsurface canopy.

5. A typical *Nereocystis* plant presents 70 square feet and a *Pterygophora* plant 18 square feet of light absorbing area (on one side).

6. The most abundant large invertebrates are an anemone, *Anthopleura xanthogrammica,* the gum boot chiton, *Cryptochiton stelleri,* and the bat star, *Patiria miniata.*

7. *Laminaria* and *Pterygophora* stipes and holdfasts provide important microhabitats for many small animals.

8. Large urchins, *Stronglyocentrotus franciscanus,* are absent. California sea otters are known to prey heavily upon this species, probably accounting for its local extinction. I suspect that the absence of this urchin allows the luxuriance of algae and sessile fauna that is observed.

9. A total of 248 species from the sublittoral zone in this locality is listed.

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EFFECTS OF X-IRRADIATION UPON POSTNATAL GROWTH IN THE MOUSE[1]

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Relatively little attention has been directed towards the long-term effects of embryonic or fetal irradiation on the subsequent vigor and well being which the irradiated may attain in later life. However, since an individual's development, in a sense, unfolds from conception until death, it was felt desirable to investigate systematically effects of *in utero* irradiation upon postnatal development. The present study has emphasized effects on development after parturition in mice, as measured by such responses as growth from birth to maturity, lifetime fecundity, and total lifespan, of which the growth effects will be reported in this paper.

The mammalian embryo is in a unique stage of development because of the great number of cells that are actively undergoing differentiation. Radiant energy absorbed during a period of development may act as an agent directing the organism's development into new paths. The redirection may stimulate either nuclear changes which have permanent continuity in later cell generations or cytoplasmic influences which may possibly be more transient. Of the various agents which may make these changes, ionizing radiations are especially useful since their penetrant actions have a general distribution throughout the entire organism. Patterns of sensitivity which are characteristic of the embryo may be revealed, therefore, by the selective response of the exposed structures.

Numerous congenital malformations have been reported as a result of embryonic radiation. Most of the earlier works are difficult to interpret since careful control of the embryo's age at irradiation was not made and the physical factors of radiation often were not standardized. These changes involved many organs of the developing individual. They indicate well defined critical periods during which the cells are susceptible to redirection of development. They further indicate that the susceptibilities change as development progresses, making some elements more resistant and causing other elements to enter more sensitive periods. In general for any one type sensitivity the periods appear to be quite restricted in the length of time over which they are active. There is furthermore a dose-dependence. Measurement of these effects has largely been confined to the observations made on the young prior to parturition or but a few days thereafter. Long-term effects have scarcely been considered.

An additional major shortcoming of many of the earlier experiments is the fact that the animals used were either animals of unknown heterogeneous origin

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or else animals all of uniform origin. In this study several genetic backgrounds were utilized in order to obtain information on important genotypic-environmental interactions. The use of several different genotypes makes it possible to draw more valid inferences from a single experiment to an over-all population of animals than if the results were gained from a single genetic background. The effects of *in utero* irradiation upon postnatal development in this study have been measured in three inbred strains of mice and all their possible hybrids.

Materials and Methods

The mice used in this investigation were taken from three inbred strains maintained at the Genetics Laboratory of Iowa State University. The strains were designated by the Committee on Mouse Nomenclature as BALB/Gw, K, and S. The strains were differentiated originally by resistance to mouse typhoid (Gowen, 1948), but are known to differ in a number of other physiological characters, including differences in response to various effects of irradiation (Grahn, 1954; Gowen and Stadler, 1956; Stadler and Gowen, 1957a, 1957b). The three strains cover a wide range of the spectrum of radiation response, the Ba and K strains representing relatively susceptible strains and the S strain representing a relatively resistant strain.

Animals that were to be irradiated as embryos were obtained exclusively from first litters of matings within and among the three inbred strains, including reciprocal matings. Females were examined daily for the presence of a vaginal plug, this plug being the sole criterion used to time the period of gestation and the approximate age of embryos at irradiation. Although the majority of matings in mice take place during the night, they have been known to occur throughout the entire 24-hour period. In this experiment, nevertheless, all mice were considered to have mated during the same time of night. The time of 4:00 AM was chosen as the approximate time of fertilization since Snell (1940) determined that in the Bagg strain of mice the modal ovulation period was between midnight and 3:00 AM, with fertilization occurring shortly after liberation of the egg. All pregnant females were irradiated at about 4:00 PM so that the embryological ages at irradiation were approximately $6\frac{1}{2}$, $10\frac{1}{2}$, $14\frac{1}{2}$, and $17\frac{1}{2}$ days. These embryological ages were chosen since they represent, in the mouse, developmental stages which correspond to a period shortly after implantation, a period during the height of major organ formation, a period of minor organ formation, and a period concerned with growth of the fetus.

The experiment was designed as a factorial with three strains of mice and all their possible hybrids, making a total of nine different inheritance types, and five levels of irradiation including 0, 20, 80, 160, and 320 roentgens.

Pregnant females were examined in the morning and in the evening, so that newborn litters usually were found within 12 hours of birth. Mice in a litter were marked individually at birth by means of india ink injected from a hypodermic needle subcutaneously. Individuals were weighed at birth, 12, 26, 40, 60, and 75 days of age. Birth weights were recorded to the nearest hundredth of a gram, and all other weights to the nearest tenth of a gram. All litters were weaned at 30 days, and the males and females separated at this time.

Within each dose-embryological age treatment a minimum of two males and two females of each inheritance type was sought by 75 days. The experiment had unequal subclass numbers, due to differential litter sizes and differential postnatal viability. It was necessary, therefore, to use disproportionate frequency analyses of all the data because of the unequal subclass numbers. The statistical procedures used in the analysis of the data are essentially as those described by Snedecor (1956), and additional details will be explained with the presentation of results.

In addition to the irradiation of embryos, a second phase of the experiment was undertaken in order to determine effect of x-irradiation upon newborn litters. It was hoped that this aspect of the experiment would also help to elucidate some of the radiation effects that were due to direct effects on the embryos and other effects that may have been mediated through the maternal organism. Litters were irradiated at 4:00 PM on the day they were born. The dam did not receive any irradiation. The same strains of mice and the same levels of radiation were used in this study. Both experiments were conducted in a well-ventilated room, in which the environment and management were relatively constant. Food and water were provided *ad libitum.*

The source of irradiation was a General Electric Maxitron which operated at 250 pkv, 30 ma with 0.25 mm. Cu + 1 mm. Al filtration at a distance of 50 cm. from anode to mid-mouse. The dose rate was approximately 133 r/minute, the dosage rates having been measured in air by means of a rate meter. Pregnant mice were exposed to single doses of whole-body irradiation within a circular, wooden container, 6½ inches in diameter, and 1 inch in depth. The base of the container was ¼- by ¼-inch wire mesh, and the top was covered with two layers of cellophane. In the experiment in which newborn litters were irradiated, the entire litter was exposed to whole-body irradiation in small, plastic trays, and then immediately returned to their dam.

A complete description of the methods and results of this investigation may be found in a doctoral dissertation (Nash, 1960) on file at the Iowa State University Library.

RESULTS

Irradiation of mouse embryos

In the results that follow the two sexes are treated separately, in accordance with the generally observed fact that male mice grow more rapidly than females. In interpreting the data it was also necessary to take into account the fact that growth in the mouse is known to be affected by litter size. Individuals from smaller litters tend to grow faster than individuals from larger litters. The body weight data in this study supported this conclusion. The effect of litter size or weight was not of primary interest in this experiment, and also added an unnecessary complication in the interpretation of the main effects. This variable could not be controlled experimentally but could at least be standardized by a statistical adjustment of the data. Consequently all weights from birth to 75 days utilized in the analysis were adjusted for litter size at birth. A litter size of nine, which represents the mean over-all treatments, was used as the base point in adjusting for litter size. The regressions of body weight on litter size at birth were calculated for each treatment. Some heterogeneity between regression coefficients of the different treatments was noted. However, it seemed clear that the use of the mean regression coefficient of all treatments for adjusting the body weights would be the most adequate means of standardizing the data.

Radiation response of males

The body weight means and standard errors for the male progeny are shown in Table I. It is evident that body weight response to *in utero* irradiation is dependent both on the level of irradiation and on the age of the embryo when irradiated. The effects of irradiation on postnatal viability will be presented in a future paper, but it is evident from Table I that there may be considerable differential postnatal mortality, as seen in the absence of any embryos that received 160 r or 320 r at 10½ days surviving to 12 days post-partum.

Table I

Body weight means and standard errors for male progeny; all weights adjusted for litter size at birth

Embryological age at irradiation	Irradiation dose	Age postparturition in days					
		Birth	12	26	40	60	75
Control	0 r	1.40 ± .02	5.0 ± .1	8.9 ± .4	17.7 ± .4	23.1 ± .3	24.7 ± .3
6½ days	20 r	1.36 ± .02	5.3 ± .2	9.7 ± .4	19.1 ± .4	23.8 ± .4	25.9 ± .4
	80 r	1.36 ± .02	5.5 ± .2	10.2 ± .4	19.2 ± .5	24.1 ± .3	25.9 ± .3
	160 r	1.35 ± .02	6.1 ± .2	11.3 ± .3	20.1 ± .5	24.6 ± .4	26.7 ± .4
10½ days	20 r	1.39 ± .02	5.5 ± .1	10.8 ± .3	20.1 ± .3	24.6 ± .3	25.9 ± .4
	80 r	1.25 ± .01	5.1 ± .1	9.1 ± .3	18.1 ± .3	21.7 ± .3	23.0 ± .3
	160 r	0.97 ± .02	—	—	—	—	—
	320 r	0.61 ± .02	—	—	—	—	—
14½ days	20 r	1.37 ± .01	4.9 ± .1	9.3 ± .3	18.7 ± .3	23.4 ± .3	24.7 ± .3
	80 r	1.31 ± .02	4.9 ± .2	8.9 ± .3	17.4 ± .7	22.2 ± .6	23.6 ± .6
	160 r	1.25 ± .02	4.9 ± .1	7.3 ± .3	14.5 ± .4	19.7 ± .4	21.3 ± .3
	320 r	0.98 ± .02	4.1 ± .5	5.8 ± .6	7.6 ± 1.0	8.8 ± .1	9.1 ± .2
17½ days	20 r	1.39 ± .01	5.1 ± .1	9.2 ± .3	18.0 ± .6	23.7 ± .4	25.3 ± .4
	80 r	1.35 ± .02	5.0 ± .2	9.2 ± .6	17.5 ± .6	23.2 ± .4	24.6 ± .4
	160 r	1.33 ± .02	4.7 ± .1	8.4 ± .4	16.0 ± .6	21.0 ± .3	22.1 ± .3
	320 r	1.32 ± .02	4.1 ± .1	6.6 ± .3	12.2 ± .7	17.1 ± .6	18.6 ± .5
Newborn	0 r	1.35 ± .02	5.3 ± .1	9.5 ± .3	17.9 ± .4	23.7 ± .3	25.4 ± .3
	20 r	1.37 ± .01	4.7 ± .2	9.1 ± .4	17.9 ± .6	22.6 ± .4	24.1 ± .4
	80 r	1.36 ± .01	5.3 ± .1	9.3 ± .3	17.8 ± .4	22.1 ± .3	23.6 ± .3
	160 r	1.35 ± .01	4.7 ± .2	8.3 ± .4	15.3 ± .7	20.1 ± .8	21.7 ± .6
	320 r	1.37 ± .01	4.2 ± .2	6.6 ± .3	12.4 ± .5	16.8 ± .5	18.8 ± .5

Irradiation at 6½ days

Irradiation at 6½ days with doses up to 160 r apparently had no deleterious effect on postnatal growth. However, no litters were born to any of nine females that had been exposed to 320 r at this stage of pregnancy, indicating that this dose may cause a 100% prenatal loss of progeny. Embryos that had received 160 r even showed an accelerated growth compared to controls. A significant difference in body weights was observed by the twelfth day and continued through 75 days. The relative difference reached a maximum at 26 days (27%) and appeared to be leveling off at about 8% at 75 days.

TABLE II

Body weight means and standard errors for female progeny; all weights adjusted for litter size at birth

Embryological age at irradiation	Irradiation dose	Age postparturition in days					
		Birth	12	26	40	60	75
Control	0 r	1.32 ± .01	4.9 ± .1	8.0 ± .3	15.9 ± .4	19.9 ± .3	21.1 ± .3
6½ days	20 r	1.28 ± .03	5.4 ± .2	10.0 ± .4	16.9 ± .6	20.7 ± .3	21.9 ± .3
	80 r	1.35 ± .01	5.1 ± .1	8.9 ± .3	16.4 ± .3	20.0 ± .2	21.5 ± .2
	160 r	1.23 ± .02	5.8 ± .2	10.8 ± .4	17.9 ± .5	21.2 ± .5	21.6 ± .4
10½ days	20 r	1.33 ± .02	5.5 ± .1	10.1 ± .3	17.9 ± .3	20.9 ± .4	22.0 ± .3
	80 r	1.21 ± .01	5.3 ± .2	9.0 ± .3	15.8 ± .3	18.4 ± .4	19.6 ± .4
	160 r	0.97 ± .02	—	—	—	—	—
	320 r	0.57 ± .02	—	—	—	—	—
14½ days	20 r	1.32 ± .01	4.8 ± .1	8.7 ± .2	15.8 ± .2	19.0 ± .2	20.6 ± .2
	80 r	1.31 ± .02	4.9 ± .1	9.2 ± .2	16.5 ± .3	19.6 ± .3	20.5 ± .3
	160 r	1.15 ± .01	4.4 ± .1	6.3 ± .2	12.0 ± .3	15.5 ± .3	16.6 ± .3
	320 r	0.96 ± .01	5.5 ± .2	6.8 ± .1	8.9 ± .9	10.5 ± .6	12.0 ± .0
17½ days	20 r	1.36 ± .01	5.2 ± .1	9.7 ± .3	17.1 ± .3	20.2 ± .2	21.8 ± .2
	80 r	1.31 ± .02	5.2 ± .2	9.2 ± .4	16.4 ± .4	20.0 ± .3	21.0 ± .3
	160 r	1.30 ± .02	4.8 ± .1	8.4 ± .4	14.4 ± .4	17.8 ± .3	18.8 ± .3
	320 r	1.25 ± .01	3.9 ± .1	6.4 ± .3	10.5 ± .5	14.4 ± .5	15.7 ± .5
Newborn	0 r	1.30 ± .01	5.3 ± .1	9.5 ± .4	16.6 ± .4	19.9 ± .3	20.8 ± .4
	20 r	1.32 ± .01	4.8 ± .2	8.5 ± .3	15.9 ± .3	18.9 ± .3	19.6 ± .4
	80 r	1.33 ± .01	4.9 ± .1	9.1 ± .3	15.7 ± .3	18.4 ± .3	19.6 ± .3
	160 r	1.34 ± .01	4.8 ± .2	8.2 ± .4	14.5 ± .4	16.8 ± .3	17.8 ± .3
	320 r	1.32 ± .01	4.3 ± .1	6.5 ± .2	11.3 ± .4	14.2 ± .3	15.4 ± .3

Irradiation at 10½ days

Doses of 80 r and above had noticeable effects on growth when given at 10½ days, resulting in lowered body weights at birth. After a dose of 320 r birth weights were less than half of those of controls. Embryos exposed to 160 r or 320 r were stillborn or died within a few days of birth. Although mice that had been exposed to 80 r weighed less than controls at birth, the survivors could not be distinguished from controls again until 60 days after birth. By 75 days the difference amounted to 7%.

Irradiation at 14½ days

Differences in birth weights were apparent after irradiation with 160 or 320 r. However, by the twelfth day of observation all irradiated groups were indistinguishable from controls. By 26 days of age animals that had received 160 r or 320 r were significantly lighter than controls. This pattern held throughout the rest of the period of observation, 160 r progeny weighing 89%, and 320 r progeny only 37% of controls at 75 days.

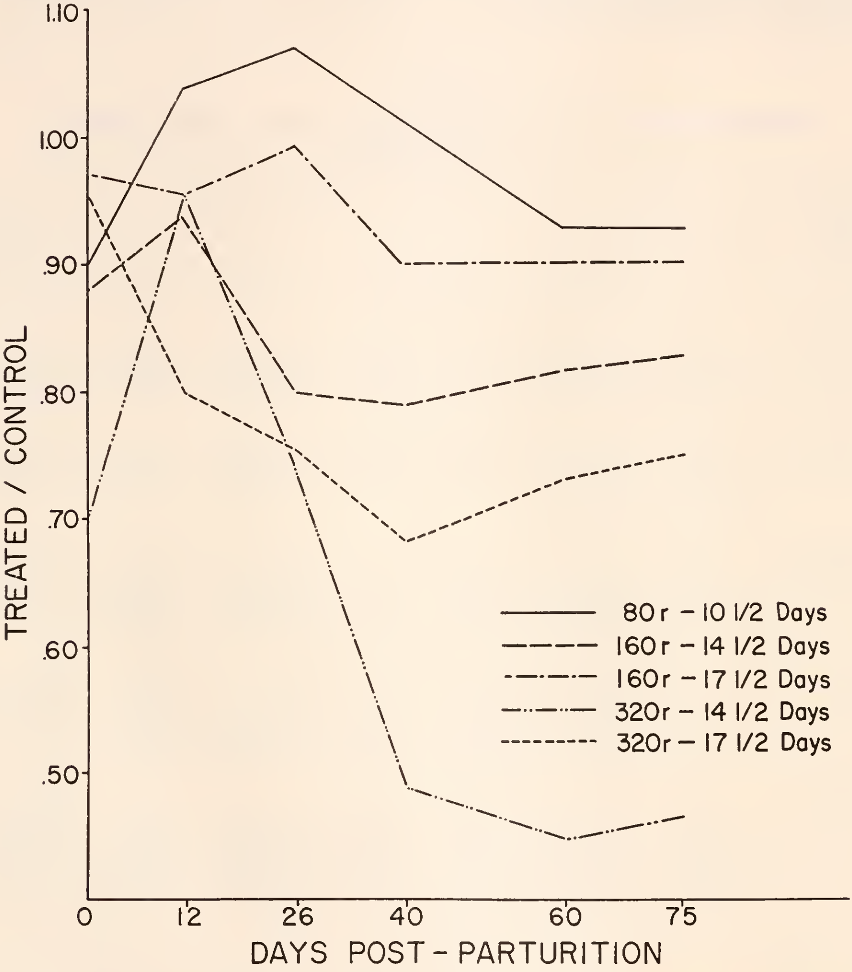

FIGURE 1. Ratio of the treated body weight means to control body weight means.

Irradiation at 17½ days

Following irradiation at 17½ days there was no significant effect on birth weights. By 12 days 320 r progeny had a lower weight than controls, and remained lower, reaching a minimum at 40 days when they were only 68% of control weights, and recovering to only 75% by 75 days. A difference between 160 r progeny and controls became evident at 60 days and continued to 75 days. Doses of 20 r or 80 r apparently did not produce significant changes at any period.

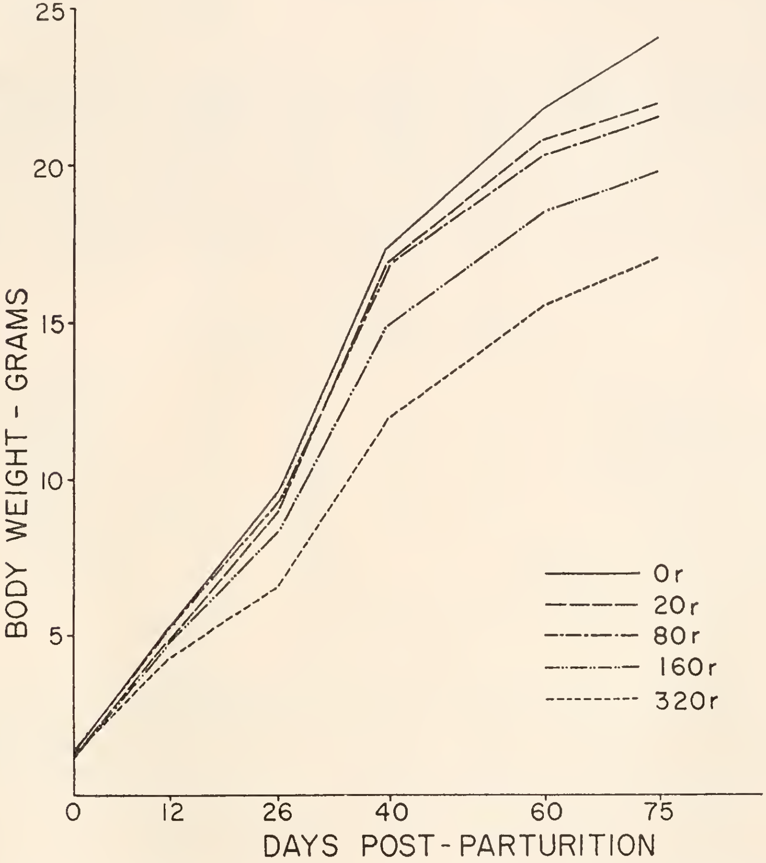

FIGURE 2. Irradiation of newborn mice. Unweighted means of male and female mean weights for each level of irradiation.

Radiation response of females

The mean body weights of the female progeny are shown in Table II. Examination of these data shows that the general body weight response of the females to embryonic irradiation was similar to that of the males. At 75 days of age the same treatments that produced significantly lower body weights in the males have produced significantly lower body weights in the females. These treatments were 80 r at 10½ days, 160 r at 14½ and 17½ days, and 320 r at 14½ and 17½ days. It is of interest to examine these treatments more closely over the period from birth to 75 days. For this purpose the ratio of the Mean Treated Weights/Mean Control

Weights has been calculated for each of these treatments by using the unweighted mean of the male and female mean weights. The results are presented in Figure 1. In general, those progeny that show a postnatal growth depression do not show the maximum response to irradiation until 40 days or more after birth. In addition, there appears to be little recovery by 75 days.

Irradiation of newborn mice

The experiment in which newborn litters were irradiated was begun almost one year after the start of the *in utero* experiment and includes its own group of untreated or control litters. The body weights in this experiment were also adjusted for litter size at birth. The birth weights in this case represent weights taken before treatment. The body weight means and standard errors for male and female progeny are given in Tables I and II, respectively, and the unweighted mean of the male and female weights illustrated in Figure 2. The general response of animals irradiated at birth closely follows that of animals irradiated as $17\frac{1}{2}$-day embryos. Progeny that were exposed to 320 r are already 20% lighter than controls by 12 days, and, after dropping to 31% at 26 and 40 days, are still 26% lighter at 75 days. Mice given 160 r at birth are significantly lower than controls by 26 days. There is some evidence that newborn litters are more affected by irradiation than $17\frac{1}{2}$ day embryos, as demonstrated by the fact that after a dose of 80 r to newborn progeny, a significant weight change was observed by 60 days of age and was continued through 75 days.

TABLE III

Breakdown for the statistical analysis

Source of variation	d.f.	Components of variation
Among genotypes	8	$E + 2_j G$
Among dosages	(j-1)*	$E + 18 T$
Genotype × dosage	8(j-1)	$E + 2 GT$
Between sexes	1	$E + 9_j F$
Sex × genotype	8	$E + j FG$
Sex × dosage	(j-1)	$E + 9 FT$
Sex × genotype × dosage	8(j-1)	$E + FGT$
Unaccounted for variation		E

* j = 3, 4, or 5, depending on which embryological age is analyzed.

Estimation of components of variation

The amount of variation in body weight response to *in utero* irradiation can be partitioned additively into the amounts due to the various effects and their interactions by utilizing the estimated components of variance derived from an analysis of variance. The general mathematical model upon which the component analysis is based is:

$Y_{ijkl} = u + g_i + t_j + (gt)_{ij} + f_k + (fg)_{ik} + (ft)_{jk} + (fgt)_{ijk} + e_{ijkl}$ where $u =$ the overall mean; $i = 1, 2, \ldots, 9$, the inheritance types; $j = 1, 2, 3$, (or 4 or 5), the levels of irradiation; and $k = 1, 2$, the sexes. As there were disproportionate sub-class numbers, the method of unweighted means was used in the analysis of

TABLE IV

Components of variance for radiation treatments at different days of gestation in mice as measured by body weights at different days following birth

Components of variance effect	Day of treatment	Birth	Postpartum growth stages					Means
			12	26	40	60	75	
Heredity	6½	23.1	16.2	15.8	21.8	7.5	4.2	14.8
	10½	0.9	30.2	28.6	33.9	22.1	17.4	22.2
G	14½	7.7	3.5	18.3	16.1	12.1	9.2	11.2
	17½	25.6	34.3	49.9	31.0	12.2	11.1	27.4
	birth	10.8	24.3	27.7	27.2	15.8	13.4	19.9
	Means:	13.6	21.7	28.1	26.0	13.9	11.1	19.1
Dose	6½	3.4	18.1	22.0	13.7	5.4	2.6	10.9
	10½	90.6	7.1	15.1	17.3	12.9	10.3	25.6
T	14½	76.9	2.7	23.8	31.7	25.8	25.4	31.1
	17½	5.9	20.6	15.6	39.9	40.5	43.2	27.6
	birth	.3	18.0	29.0	41.1	36.7	33.0	26.3
	Means:	35.4	13.3	21.1	28.7	24.3	22.9	24.3
Heredity × Dose	6½	32.1	53.3	52.6	30.8	17.5	7.3	32.3
	10½	6.1	49.0	48.1	14.9	9.6	6.9	22.4
GT	14½	7.4	64.7	43.8	21.9	10.0	8.4	26.0
	17½	42.4	32.1	27.8	12.4	10.1	5.1	21.7
	birth	51.6	43.5	32.8	13.1	5.3	5.4	25.3
	Means:	27.9	48.5	41.0	18.6	10.5	6.6	25.5
Sex	6½	15.0	1.7	1.1	22.8	60.2	78.2	29.8
	10½	.6	0	.4	22.8	49.1	58.9	22.0
F	14½	1.5	4.4	0.3	12.0	38.0	44.0	16.7
	17½	9.5	.2	.4	7.8	29.3	35.0	13.7
	birth	6.8	0	.1	8.5	34.5	42.4	15.4
	Means:	6.7	1.3	.5	14.8	42.2	51.7	19.5
Heredity × Sex	6½	7.2	1.2	1.4	0.9	3.0	1.2	2.5
	10½	0	0	0	0	0	0.9	.2
	14½	0	0	0	1.5	3.2	2.8	1.3
GF	17½	0	0	.2	2.0	.5	0	.5
	birth	0	0	0	.3	.8	0	.2
	Means:	1.4	.2	.3	.9	1.5	1.0	.9
Dose × Sex	6½	6.0	0	0.5	0.4	0	1.0	1.3
	10½	.1	0	0	0	0	0	0
TF	14½	1.0	0	.1	1.7	1.7	1.5	1.0
	17½	0	0	0	0	1.7	1.1	.5
	birth	0	.6	0	.3	.7	.8	.4
	Means:	1.4	.1	.1	.5	.8	.9	.6
Heredity × Dose	6½	0	0	0	0	0	0.1	.0
× Sex	10½	0	0	0	0	.4	0	.1
GTF	14½	0	0	3.9	7.0	4.7	4.3	3.3
	17½	0	0	0	1.5	2.3	1.1	.8
	birth	0	0	0	2.3	2.5	1.6	1.1
	Means:	0	0	.8	2.2	2.0	1.4	1.1

TABLE IV—(*Continued*)

Components of variance effect	Day of treatment	Birth	Postpartum growth stages					Means
			12	26	40	60	75	
Random	6½	12.3	9.5	6.6	9.6	6.4	5.4	8.3
	10½	1.7	13.7	7.8	11.1	5.9	5.6	7.6
	14½	5.5	24.7	9.8	8.1	4.5	4.4	9.5
E	17½	16.6	12.8	6.1	5.4	3.4	3.4	8.0
	birth	30.5	13.6	10.4	7.2	3.7	3.4	11.5
	Means:	13.3	14.9	8.1	8.3	4.8	4.4	9.0

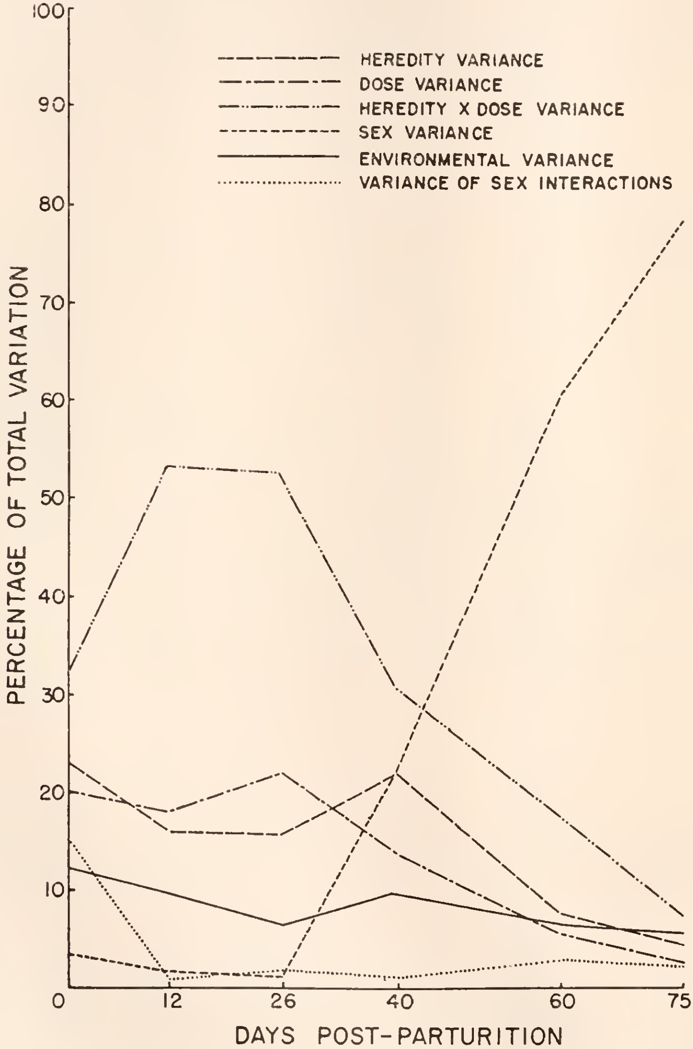

FIGURE 3. Irradiation at 6½ days. Breakdown of variation in body weight. Components expressed as a percentage of total variation.

variance. The general breakdown for the analysis is given in Table III. The components can be interpreted as follows: *G* is the variation due to genotypic or hereditary differences, *T* is the variation due to differences in effects of the dosage levels, and *F* is the variation between sexes. The interaction terms are interpreted

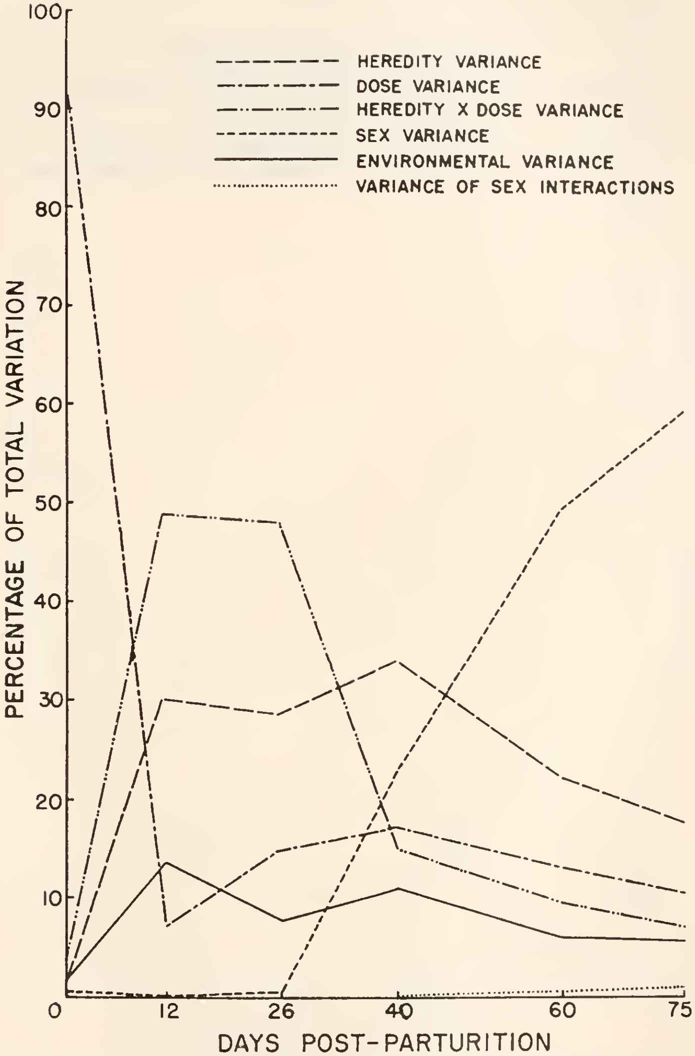

FIGURE 4. Irradiation at 10½ days. Breakdown of variation in body weights. Components expressed as a percentage of total variation.

as arising from the differential responses of the genotypes or sexes from one level of irradiation to the next. The term, *E*, is considered due to uncontrollable variation, and represents random variation of individual differences of mice of the same sex within a litter given the same treatment. An analysis of variance was obtained for each of the embryological ages separately. The results of the component analysis are shown in Figures 3–7.

Although there is considerable variation among the different embryological ages in the percentages of total variation that are attributable to the various factors operative in this experiment, there are some general patterns that hold true for all ages. Within those embryological ages where progeny show the greatest body weight response, not including birth weight response, to irradiation the effects of irradiation do not reach their maximum contribution to total variation until usually

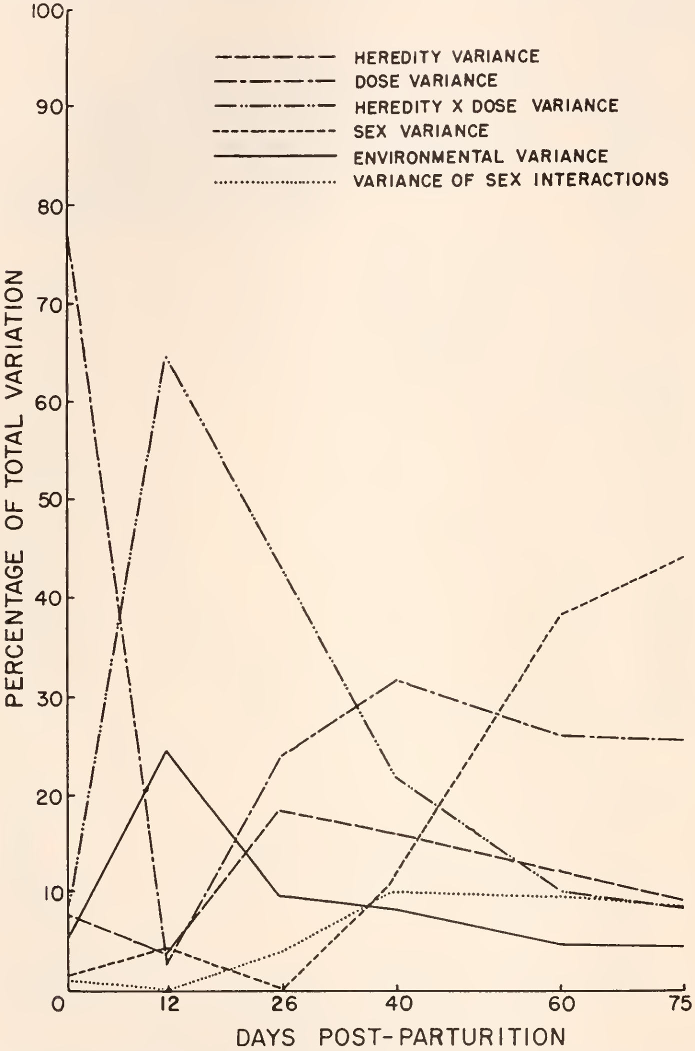

FIGURE 5. Irradiation at 14½ days. Breakdown of variation in body weights. Components expressed as a percentage of total variation.

40 days or more after birth. Furthermore, this effect declines little, if at all, by 75 days.

The heredity influences on variance (Table IV) contributed about 20% of the variances for the different growth stages as separated by the x-ray treatments at the specified embryonic stages of development. This average hereditary effect was almost identical with that observed for the mice treated directly after birth, 19.9%. The highest genotypic effects were observed for the $10\frac{1}{2}$- and $17\frac{1}{2}$-embryonic day treatments. Lower values were observed for the embryos exposed at $14\frac{1}{2}$ and $6\frac{1}{2}$

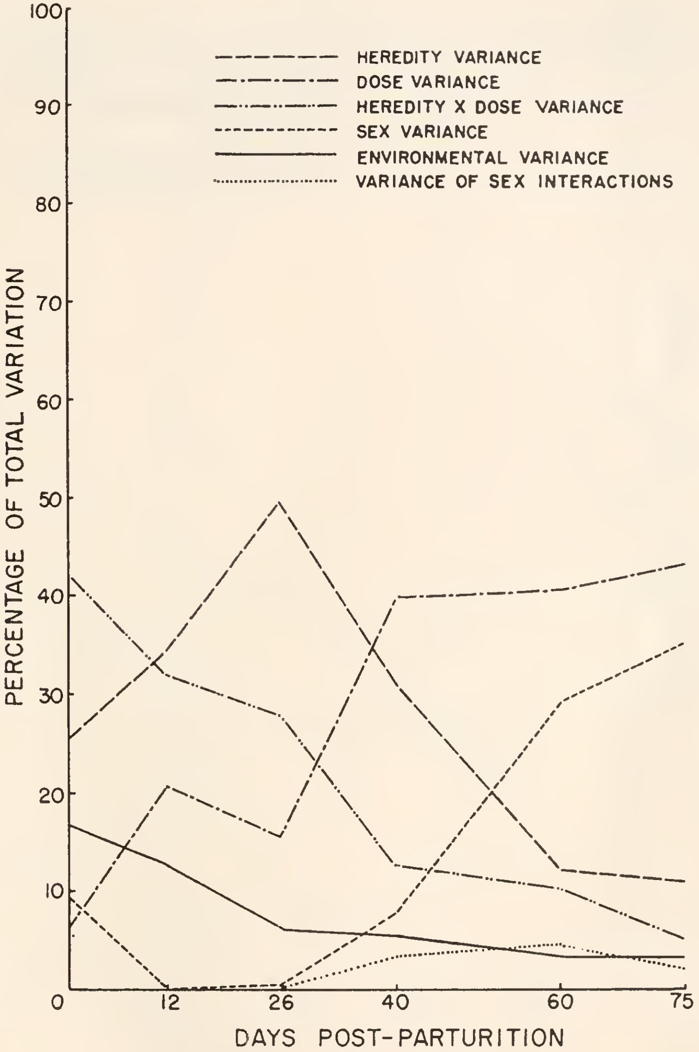

FIGURE 6. Irradiation at $17\frac{1}{2}$ days. Breakdown of variation in body weight. Components expressed as a percentage of total variation.

days after fertilization. The hereditary effects were strongest at the 12-, 26-, and 40-day growth periods. The decrease from 40 to 60 and 60 to 75 days post partum corresponded to the point in development where the infant mice were changing from their dependence on both their own and their mother's inheritances (in terms of food, milk supply) to that where their own genotypes were alone responsible for their growths. Both the day of fetal treatment effects and the time when the growth stages were measured appear to show consistent differences in the hereditary effects at about 0.01 significance (Table V). The interaction between fetal

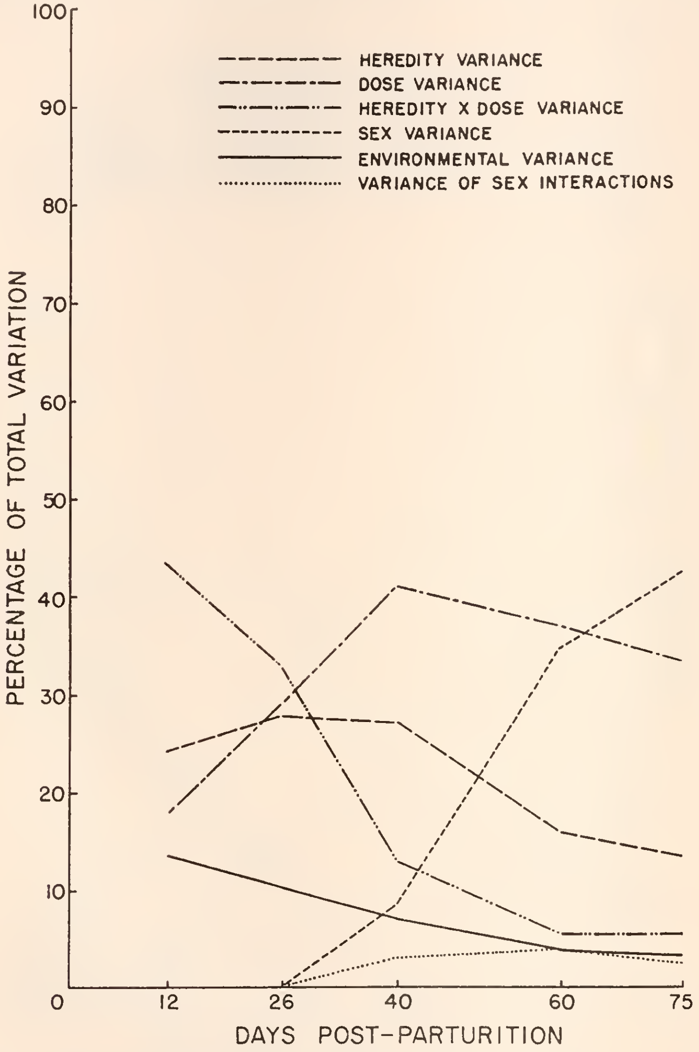

FIGURE 7. Irradiation of newborn animals. Breakdown of variation in body weight. Components expressed as a percentage of total variation.

day of treatment and the ultimate effect of the hereditary component on growth at the later period was almost significant at the 0.05 level, showing that there may be some specificity in the mode of action of the hereditary factors.

TABLE V

Analyses of variance of the components for the radiation treatments at different days of gestation as measured by body weights at different days following births

	Source of variation	d/f	M.S.
Heredity	Total	29	
	Day (D)	4	240.2
	Growth (G)	5	255.9
	D × G	20	60.9
Dose	Total	29	
	Day	4	364.2
	Growth	5	276.6
	D × G	20	473.2
Heredity × Dose	Total	29	
	Day	4	105.5
	Growth	5	1405.7
	D × G	20	130.2
Sex	Total	29	
	Day	4	256.9
	Growth	5	2434.9
	D × G	20	57.3
Sex × Heredity	Total	29	
	Day	4	5.9
	Growth	5	1.4
	D × G	20	1.9
Sex × Dose	Total	29	
	Day	4	1.6
	Growth	5	1.3
	D × G	20	1.4
Sex × Dose × Heredity	Total	29	
	Day	4	10.8
	Growth	5	4.5
	D × G	20	1.4
Random	Total	29	
	Day	4	14.7
	Growth	5	92.9
	D × G	20	31.1

The dosage of irradiation components accounted for about 25% of the average variance observed in postpartum growth. Radiation dose appeared most effective for the 14½-day and 17½-day periods. It is to be remembered, however, that the 320 r dose on the 6½-day embryos gave no progeny at birth. The effect of dose consequently was more severe at the 6½-day stage than appears from the calculated component analysis. The dose components over the different postpartum growth stages showed high variation among the different fetal treatment times. Much of the variation came at birth. Dosage effects appeared low for the 6½-day treatment. The effects were very high for the 10½- and adjoining 14½-day embryos. The nearly formed mice at 17½ days and the mice at birth were not appreciably affected. The dosage effects later in the growth period were high save for the 6½-day, and to a lesser extent for the 10½-day, fetal exposures where deaths in the 16 r and 320 r mice had been extreme. This lowering of the effects could be expected since the treatments already had removed the mice that would show extreme radiation effects, leaving in the later populations only those with lesser damage to have their growths measured. The effects of fetal day of treatment, growth stages when growth measurements were made, and their interaction, had about equal effects as judged by the component variations (Table V). They were probably all highly significant as compared with the uncontrolled variations representing the random element.

The interaction components between the hereditary elements and dosage on the particular days of fetal treatment accounted for about 25% of the variations. For data of this type this percentage was high. High specific effects between the inheritance and the ability of the fetuses to resist the x-ray irradiations at the different fetal ages are indicated. The interaction component values were comparable for the different days when the fetuses were treated. They were most significant in the early growth weights when the mice were nursing. In this period both the heredity differences of the fetuses and of their mothers were expressed. After weaning, a lowering of the interactions of heredity and dosage, as measured by the mouse weights, took place as the mouse became dependent on its own genotype.

The sex components showed variations, as would be expected, with regard to the period postpartum when they were measured. The sex effects on the weights did not become significant until the mice were aged 40 days. This is the period when puberty commences and any differential hormone secretions begin having their effects. The sex differences accounted for 40 to 50% of the variance in the weights at the 60- to 75-day periods when the chromosomal controlled sex differences were well along in their development. The variance among the components for day of treatment and sex effects had a significance of about 0.01 (Table V). The variance engendered by the differences in the growth periods when the components were measured was highly significant. The interaction between day of treatment and growth period was not significant.

The interactions of the components sex by heredity and sex by dose, and the triple interaction sex by dose by heredity, contributed but little to the total variance. Although they showed some evidence of unequal effects for day of fetal treatment they will not be discussed because of the small size of these effects.

The random or uncontrolled variances averaged about 9.0% of the total variance, a surprisingly small value. The components for this random variation are rather consistent for the days when the fetuses were treated. They show significant decreases in value from the birth to the 75-day postpartum weights. These changes may in part be due to the large variations that may be induced by the differences in quantity of milk contained in the stomachs of the just-born mice as contrasted with mice of older ages.

The variance analyses of the component values shown in Table IV are presented in Table V.

Discussion

The observations herein presented confirm as well as extend the conclusions that may be derived from the results of some of the earlier investigations in this field. Job *et al.* (1935) were the first workers to demonstrate clearly the presence of well-defined critical embryological periods susceptible to x-ray irradiations, as measured by morphological abnormalities, although earlier workers such as Von Hippel and Pagenstecher (1907) and Kosaka (1927, 1928) observed malformations following embryonic irradiations. Job *et al.* found that pregnant rats irradiated with 35 to 90 r between the eighth and eleventh day of gestation delivered abnormal embryos. The critical periods established for certain defects appeared to be: hydrocephaly, ninth day; eye defects, tenth day; and jaw abnormalities, eleventh day.

Russell (1950, 1956) studied gross visceral and skeletal malformations induced in mouse embryos following irradiation with doses of 100 r to 400 r between ½ and 13½ days of gestation. She found a wide variety of abnormalities including microphthalmia, polydactyly, limb deformities, coloboma, vaulted cranium, spina bifida, imperforate anus, tail abnormalities, hydronephrosis, and open eyelids. Using the criteria of prenatal mortality and abnormalities at birth, Russell found that the prenatal development of the mouse was divisible into three broad phases:

The pre-implantation period (½–4½ days). Irradiation during this period with x-ray doses of 100 r–200 r gave a high incidence of prenatal death, but virtually no abnormalities among those embryos surviving to term.

The period of major organogenesis (5½–13½ days). Irradiation during this period with doses up to 400 r gave almost no prenatal loss of embryos, but did cause a high incidence of malformations at birth.

The period of the fetus (14½ days to birth). Irradiation during this period of growth and minor organogenesis did not cause prenatal deaths nor any gross abnormalities at birth, although several types of abnormalities have been observed to occur later in life.

There have been few quantitative experiments concerned with the effects of embryonic irradiation upon postnatal growth. Cohn (1907) and Hanson (1923) reported reduced body size in adult stages in the rabbit and rat, respectively, but details as to levels of irradiation and age of embryos when irradiated are lacking.

Russell (1950) in an extensive experiment found that the mean birth weights of mice that had been irradiated between 8½ and 13½ days were considerably lower than those of the controls. The most critical period for both 200 r and 300 r appeared to be between the 10½- and 11½-day stages. The mean weight at 11½ days for the 200 r dose was only two-thirds of that of the controls. The work on the 11½-day stage was extended by Russell, Russell and Major (1951), and showed that points for different doses and for the controls fell on an approximately straight line, with weight reduction per 100 r averaging 0.22 gram over the three available intervals.

Wilson *et al.* (1953) found that seven embryos that had received 100 r on day 10 weighed on the average 10% less than controls at birth, but recovered this initial weight deficiency by 50 or 60 days postpartum. Ershoff and Bavetta (1958), however, observed no difference in average weights of rats at birth or at 21 days following irradiation with 150 r on days 10 or 14. Graham *et al.* (1959), using the same treatments and the same strain of rats, did find a 10% weight decrease at birth, but surviving rats were "normal" at weaning.

Levy *et al.* (1953) irradiated mouse embryos of 15½ days with 300 r, and examined the femur, mandible, and parietal bones at birth and various other days to 240 days of age. Irradiated embryos were born with bones having dimensions smaller than normal, and there were significant differences between averages on various days for both control and irradiated animals for all measurements between one and 29 days postpartum. In general, irradiated animals maintained smaller bone dimensions compared to unirradiated, although differences were not as marked as time went on.

The sampling of the entire gestational period by observing effects of x-irradiation given at four different embryological ages, namely, 6½, 10½, 14½, and 17½ days, in the present study did indicate "critical periods" for the induction of changes in postnatal growth. These critical periods are in agreement with those using body weight at birth as a criterion, as found by Russell (1950), who observed that day 11½ was close to the stage of maximum susceptibility for growth retardation. The embryological ages in order of increasing response to birth weight depression in the present study were found to be 6½, 17½, 14½, and 10½ days. The same order of sensitivity was shown in postnatal growth, if allowance is made for the fact that no progeny at all survived irradiation with 160 r or 320 r at 10½ days. This stage was the only stage in which a dose of 80 r had an effect on postnatal growth, lowered body weights having been observed by 60 days postpartum after irradiation with 80 r at 10½ days whereas 80 r at other ages did not affect body weight.

In evaluating effects of *in utero* irradiation it is necessary to consider the possible role of the maternal organism in producing abnormal development in the embryo. The evidence of various workers, including Russell (1950), Hicks (1950), Wilson and Karr (1951), Brent (1957), and Grayevsky *et al.* (1959), indicates that the role of the maternal organism is of no consequence or of very little consequence in producing morphological abnormalities observable at birth. The influence of the irradiated maternal organism on postnatal growth has not been explored adequately. Russell (1950) found that mortality after birth was apparently not due to inability of mothers that had been exposed to whole body irradiation to care for the progeny, since irradiated females were able to raise young mice to weaning age when they were given non-irradiated newborn litters to foster. No mention was made of body weights of these foster litters.

Rugh (1956) studied effects on growth of suckling young from irradiation of lactating mothers. Female mice were irradiated two days after delivery of litters. An increased retardation of growth was found in the young. There was considerable variation in results between separate experiments, making it difficult to generalize the quantitative effects of maternal irradiation on the growth of young.

Neither of the above examples provides information on direct effects of *in utero* irradiation upon postnatal growth and the indirect effects through changes in lactating ability of the mother when both of these factors are operative at the same time. The following points in the present experiment provide some indirect evidence that indicates most of the effects of postnatal growth are due to direct effects on the irradiated embryo.

Irradiation of newborn progeny without the maternal organism receiving any irradiation at all still resulted in severe disturbances in postnatal growth. A dose of 320 r given to young at birth produced body weight changes which were similar in magnitude to those obtained after a dose of 320 r at 17½ days gestation.

After irradiation with 20 r or 80 r at any of the embryological ages, and 160 r at 6½ days, postnatal growth was normal, indicating that these dose-embryological age combinations were ineffective in affecting the subsequent lactation of the mother.

In those treatments that yielded significantly lower body weights the maximum effect was not reached until weaning or later, and there was little recovery even by 2½ months of age. It appears from these results that during the early phases of postnatal growth, radiation-induced damage in the young is compensated somewhat by the nutrition furnished by the maternal organism. However, as the young shift more towards solid food and are weaned, they become completely dependent upon their own physiological systems. It is then that the direct effects of radiation may become most noticeable.

It appears for these reasons that the effects of *in utero* irradiation upon postnatal growth are due, for the most part, to direct effects of the radiation upon the embryo, and that irradiation of pregnant females has little effect on the lactation of these females. The possibility should not be excluded, however, that some specific dose-embryological age combination may have an effect on lactation which would be reflected in the postnatal growth of the young.

It is important in evaluating effects of *in utero* irradiation to consider the time at which observations are made. Thus, in some of the treatments which affected postnatal growth the most, it was not yet apparent by birth or even 12 days that growth would be retarded. It is evident in these cases that there had not been sufficient time for this type of radiation damage to have been expressed. An assessment of radiation-induced changes should always specify the criteria that are being used to determine damage.

It is of some interest to examine the similarities between these radiation-induced growth changes and growth changes effected by mutant genes, although as Russell (1954) has emphasized, it is unlikely that gene action should parallel exactly the pattern of radiation response. The two best known mutants which affect growth in the mouse are pituitary dwarfism and pygmy. There are some striking dissimilarities between effects of these genes. Pituitary dwarfism, originally described by Snell (1929), causes practical cessation of growth at 14 days postpartum. The primary effect of the gene appears to be on the anterior lobe of the pituitary.

Pygmy, first described by King (1950), is apparently not due to a lack of growth hormone, and reduction is already manifest by birth. King concluded that it is possible that the effect of this mutant is to reduce the responsiveness of tissues of the body to the growth component of pituitary hormone.

Additional types of dwarfism in the mouse include a type described by Strong (1948) which is apparently different from both pituitary dwarfism and pygmy in that affected animals are not only small at birth but also exhibit restlessness. More recently Schaible and Gowen (1961) have described a new dwarf mouse, which, although it is phenotypically similar to the pituitary dwarf, has been found not to be allelic.

It is evident from these examples that the mutant forms of dwarfism have a wide variation in the way they affect growth. In the present experiment in which entire embryos were exposed to irradiation, it appears likely that in those treatments in which weight depression was observed by birth, the result has been due to effects of various cells throughout the body which produce metabolic derangements interfering with normal assimilation and growth, and/or to effects on the pituitary gland or other growth-controlling organs which could have affected growth.

In those treatments with which there was a considerable growth retardation some time after parturition, the general growth curve and response were somewhat similar to that produced by pituitary dwarfs, although this does not mean that the pituitary gland may have been affected to some degree. The channels through which growth may be regulated are numerous, and some of the growth retardation probably is also due to direct effects of radiation on other tissues, as well as to effects on the pituitary and/or a number of other secretory glands. It will be seen in a subsequent paper that some of the treatments produced progeny which were not only stunted, but also had an increased mortality rate throughout life, a higher incidence of cataract formation, and decreased fertility.

It was seen earlier that there were considerable genetic differences in susceptibility to radiation-induced growth changes. It is likely that these differences in response are a result, in part, of differences in developmental rates of the various genotypes. Even a small difference in developmental ages during a time of rapid differentiation at the time of radiation exposure could result in a large difference in subsequent development. In addition, body weight response may be influenced by genetic variation in response to the secondary effects of radiation. Grahn (1954) determined body weight changes in six inbred strains of mice irradiated at 40 days of age, and observed clearcut genetic differences. His study is of particular importance to the present investigation since it included the Ba and S strains used in the present study. Grahn found that there were not only differences in the maximum body weight loss following radiation exposure, but that there were also genetic differences in the rate, time, and completeness of recovery from radiation effects. Comparisons between a susceptible (Ba) and resistant (S) strain indicated strain differences even after a low dose of 20 r. All of the strains showed a similar dose-response curve, indicating that the basic response was similar in the six strains. Genetic differences were being expressed through the rate of recovery from disturbed physiological activities. Due to the overall size of the present experiment the number of observations for any single genotype for any one treatment was of necessity small, and precludes the making of meaningful comparisons of individual genotypes. Nevertheless, it appears that the genetic differences between strains are influencing the different radiation effects on growth following irradiation of embryos. These genetic differences may be exerted through the abilities of individual cells and organs to resist the deterimental effects induced by radiation. Mice of certain genotypes may be able to repair damage and return to normal physiological activity more quickly than mice of other genotypes.

SUMMARY

1. The effect of x-irradiation of mouse embryos upon their postnatal development was measured by several responses: body weight changes from birth to maturity, lifetime fecundity, and total lifespan. The body weight responses are re-

ported in this paper. Three genetically differentiated inbred strains of mice, Ba, K, and S, and all their possible hybrids, including reciprocals, were used. Pregnant females were exposed to single whole-body 250 pkv x-ray dosages from 20 r to 320 r on 6½, 10½, 14½, and 17½ days gestation, as timed from the appearance of a vaginal plug. In addition the study included progeny irradiated on the day of birth without any irradiation of the maternal organism. Postnatal growth was followed from birth to 75 days of age, individuals having been weighed at birth, 12, 26, 40, 60 and 75 days.

2. Body weights were adjusted by making use of the pooled regression coefficient of body weight on litter size over all treatments. Body weight response was found to be dependent on both level of irradiation and embryological age at irradiation. The embryological ages in order of increasing sensitivity were 6½, 17½, 14½ and 10½ days. Body weight response was found also to be markedly dependent upon the age at which observations were recorded. In those treatments that produced significantly lowered body weights the maximum effect was not found usually until 40 days postpartum. Growth effects appeared to be permanent since there was little recovery evident by 75 days. Evaluation of these results emphasizes the importance of considering both immediate and delayed effects in assessing damage induced by embryonic irradiation.

3. Growth differences following embryonic irradiation were found to be under a strong genetic influence. Genetic differences in response to the induction of growth retardation were thought to be expressed as a result of genetically determined differences in recovery from disturbed physiological activities and differences in developmental age of embryos at the time of irradiation.

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TAENIOCOTYLE NOM. NOV. FOR MACRASPIS OLSSON, 1869, PREOCCUPIED, AND SYSTEMATIC POSITION OF THE ASPIDOBOTHREA

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Olsson (1869) erected the genus *Macraspis* to receive a new species, *M. elegans,* from the gall-bladder of *Chimaera monstrosa,* taken at the Skagerrack, in the North Sea. Stiles and Hassall (1908) and Neave (1940) noted that the generic name *Macraspis* Olsson, 1869 was preoccupied by *Macraspis* MacLeay, 1819 and consequently a homonym. To replace it I propose the name *Taeniocotyle* (*tainia,* ribbon, fillet; *kotylē,* cavity, acetabulum) with *Taeniocotyle elegans* (Olsson, 1869) as type species.

Taeniocotyle is a member of the family Aspidogastridae, order Aspidobothrea Burmeister, 1856. Monticelli (1892) renamed the group Aspidocotylea and Faust and Tang (1936) proposed the name Aspidogastrea. Commenting on the latter action, Hyman (1951, p. 248) remarked, "Faust and Tang have proposed a new name Aspidogastrea for the group on the ground that the name has to be derived from the genus *Aspidogaster.* This ground is mistaken. There are no rules governing the formation of names of higher taxonomic categories, and the creator of an order or class is at liberty to select any name he pleases. The author is strongly opposed to the invention of new names for groups for which names already exist." The argument against the change of name by Faust and Tang applies with equal force to the change proposed by Monticelli. Repeating a common and widely held opinion, Najarian (1961, p. 515) stated, "It is generally accepted that the subclass Aspidogastrea Faust and Tang, 1936 represents a group of worms intermediate in morphology and life history between monogenetic and digenetic trematodes." Hyman has disposed of the nomenclatorial issue and critical evaluation of data shows that the aspidogastrids are not intermediate in either morphology or life-history between the monogenetic and digenetic trematodes.

On July 13, 1961, Dr. James W. Campbell of the Rice University gave the writer a live specimen, identified as *Macraspis cristata,* from the gall-bladder of the northern stingray, *Dasyatis centrura,* taken near Woods Hole, Massachusetts. It tended to coil in spiral fashion with the adhesive organ on the external aspect. Other specimens had been taken from *D. centrura* in previous summers by members of the Rice University group at the Marine Biological Laboratory, Woods Hole. Five were made available for study by Dr. John E. Simmons, Jr., now at Emory University, Atlanta, Georgia, and three by Dr. John S. Laurie, now at the University of Utah, Salt Lake City, Utah. The latter specimens measured 218, 246,

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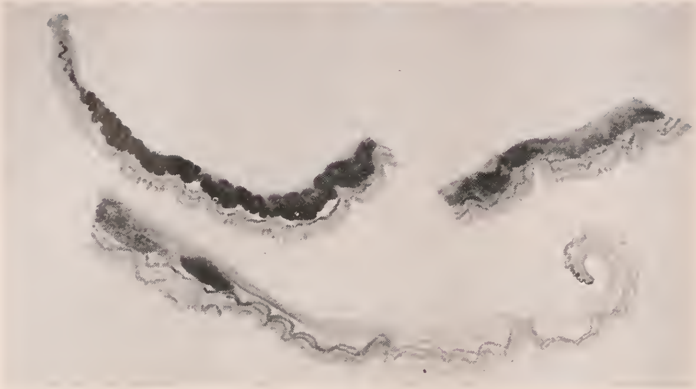

Figure 1. *Multicalyx cristata* from *Dasyatis centrura;* specimen 65 mm. long.

and 256 mm. in length. Grateful acknowledgment is made to the members of the group for their kindness and generosity. The record of collection provided by Dr. Laurie, reads:

1958—June 30, 2 specimens from the gall-bladder; after fixation, 290 and 305 mm. in length and 3 mm. in depth; the "foot" is bright red in the living worm.
1959—July 6, 2 specimens from the gall-bladder.
1959—July 8, 2 specimens from the gall-bladder.
1960—July 12, 3 specimens from the gall-bladder.
1960—July 16, 4 specimens from the gall-bladder.
1961—July 13, 1 specimen from the gall-bladder.

The specimen taken in 1961 (Fig. 1) was relaxed, flattened, fixed, stained and mounted. When compressed, the worm was flattened laterally and presents a side view of the body and a clear view of Laurer's canal and its opening on the dorsal surface. The worm is 65 mm. long and has about 400 alveoli in the "foot"

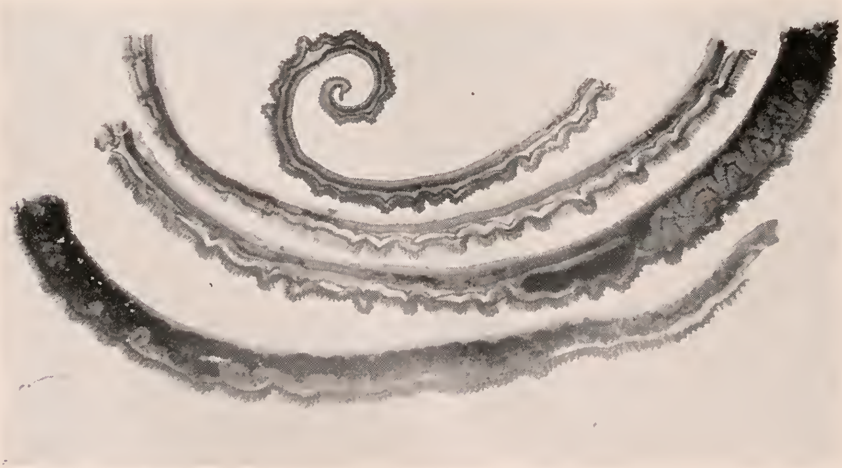

Figure 2. *Multicalyx cristata* from *Dasyatis centrura;* specimen 182 mm. long.

or adhesive organ, although they grow progressively smaller toward the posterior end of the body where new ones are added. In this worm the posterior end of the body is rounded, but in other, larger specimens the region behind the adhesive organ forms a bluntly conical tip which is turned dorsally at a right angle (Figs. 2, 3). In the worm shown in Figure 1, the pharynx is 0.56 mm. in diameter. The testis is 2.75 mm. long, 0.81 mm. in dorsoventral measurement, and slightly behind the middle of the body. The sperm duct passes forward on the ventral side of the body; at the level of the anterior end of the adhesive organ it expands to form a coiled seminal vesicle and the terminal portion of the duct, inside the cirrus sac, is surrounded by secretory cells. The cirrus sac is small, 0.40 mm. long, 0.30 mm. wide, with a relatively weak muscular wall. The common genital pore is ventral, below the posterior end of the pharynx. The ovary is ovate to pyriform, 0.48 mm. long and 0.36 mm. in dorsoventral measurement, situated about one-fourth of the body length from the anterior end. The vitellaria consist of cords of follicles which extend in the ventrolateral areas from an anterior limit about 7 mm. from the anterior end of the body to almost the posterior end. Individual follicles are spherical to oval and measure from 0.06 to 0.095 mm. in diameter. The ootype region agrees with other descriptions of the species, and Laurer's canal opens to the surface a short distance posterior to the ovary. The uterus, which courses in coils posteriad from the ootype to the level of the testis and then forward to the genital pore, is filled with an enormous number of eggs. In the initial portion of the uterus the eggs are thin-shelled and more spherical whereas in the later loops the eggs become heavy-shelled, with flattened opercular ends and

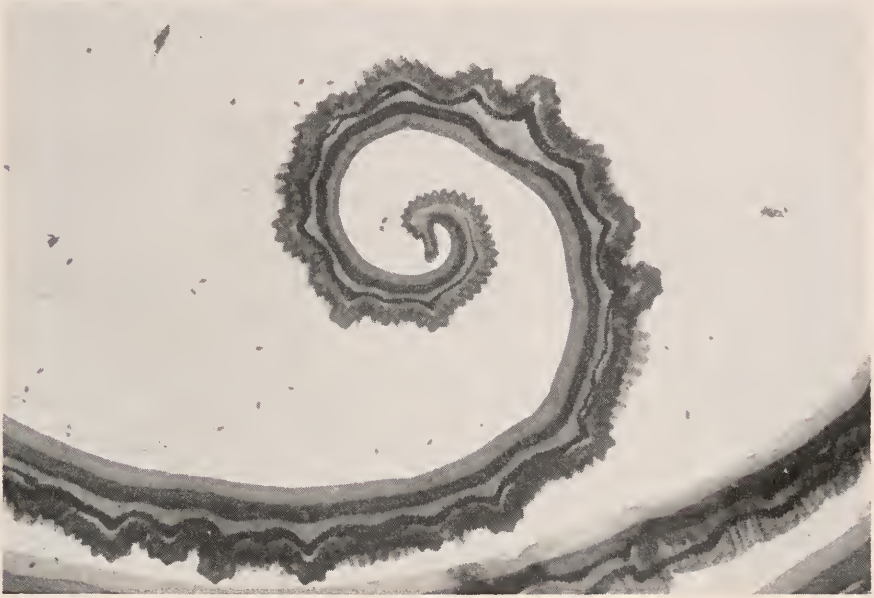

FIGURE 3. Same specimen as Figure 2; reversed in photograph, to show the posterior end of the worm. The smaller specks in the photograph are eggs of the parasite.

antopercular ends that are thickened and may bear small knobs. Eggs measure 0.12 to 0.14 mm in length and 0.062 to 0.074 mm in width.

Other specimens collected in previous years are much larger; the largest ones available for this study are 180 to 256 mm. in length. The worm shown in Figure 2 is 182 mm. long, and has over 800 alveoli. The pharynx is 0.625 mm. in diameter. The testis is 5.6 mm. long, 1.24 mm. in greatest depth, and is situated at the anterior end of the second third of the body. In the largest specimen, the testis is at the posterior end of the anterior third of the body. In the specimen shown in Figure 2, the cirrus-sac is 0.57 mm. long and 0.38 mm. in diameter. The ovary is 1.00 mm. long, 0.75 mm. deep, and is situated about one-sixth of the body length from the anterior end, about midway between anterior end and the testis. It is apparent that the gonads are shifted relatively forward as the body enlarges and increases in length and more alveoli are added at the posterior end. The eggs are somewhat larger than in the specimen shown in Figure 1, and measure 0.135 to 0.153 mm. in length by 0.080 to 0.094 mm. in width.

Dr. Laurie reported (*in littoris*) that according to Dr. James E. Lynch, "*Macraspis* is fairly abundant in the ratfish (*Hydrologus collici*) in Puget Sound."

The specimens from *D. centrura* are determined as specifically identical with the single worm reported by Faust and Tang (1936) from the spiral valve of the cow-nosed ray, *Rhinoptera quadriloba* (Le Sueur) taken in Biloxi Bay, Mississippi. Since all other individuals of this species have been found in the gall-bladder, the location reported by Faust and Tang is questionable. Faust and Tang (1936) described the worm as *Stichocotyle* (*Multicalyx*) *cristata* n. sp. The specimen was assigned to the genus *Stichocotyle* but differed in fundamental respects. So *Stichocotyle* was expanded and divided into two subgenera: *Stichocotyle,* and a new subgenus, *Multicalyx*, which was erected to receive *S. cristata*. Manter (1954) transferred the species, *cristata*, to the genus *Macraspis* and Dollfus (1956) listed *Multicalyx* as a synonym of *Macraspis*. Discussing the alteration of the generic diagnosis of *Stichocotyle* and subdivision of the genus, Brinkmann (1957, p. 14) stated, "All this systematic nonsense is accordingly useless and in fact those two characteristics: 1) the acetabular structure, and 2) the presence of a single testis, in which Faust and Tang find that their species differs from *Stichocotyle*, and for which they emend the diagnosis of the latter genus, *are just the characteristics which it shares with the genus Macraspis*." Brinkmann noted differences between *M. elegans* and *M. cristata* which preclude their identity. Referring to the account of Faust and Tang, Dollfus (1958, p. 227) stated, "L'espèce était évidemment nouvelle, mais il est incompréhensible que Faust & Tang l'aient placée dans *Stichocotyle*, alors que c'est manifestement un *Macraspis;* toutefois, Faust & Tang ont proposé un nouveau sous-genre: *Multicalyx*. Ce sous-genre a été considéré par H. W. Manter (1954, p 482), R. Ph. Dollfus (1956, p. 12), Aug. Brinkmann (1957, pp. 13, 17), comme simplement un synonyme de *Macraspis*." The citation in the title of Dollfus' (1958) paper, "*Macraspis cristata* (E.-C. Faust et C.-C. Tang, 1936) H.-W. Manter 1936," is obviously an error of transcription since Dollfus gave the correct date, Manter, 1954, in the text.

As noted, previous authors have considered *Multicalyx* to be synonymous, at least in part, with *Macraspis* Olsson, 1869. The species, *elegans*, is type of the generic concept represented by the preoccupied name *Macraspis,* now *Taeniocotyle*, while *cristata* was named type of the subgenus, *Multicalyx*. If the species *elegans* and *cristata* are regarded as congeneric, *Multicalyx* would replace *Macraspis* (preoccupied), but such action would create a vexatious nomenclatorial problem. *Multicalyx cristata* is type of the subgenus, now raised to generic rank, but the genus originally had the species *elegans* as type. A genus can not have two type species, but the differences between the species *elegans* and *cristata* are of sufficient magnitude to warrant the elevation of *Multicalyx* to generic status, with resolution of the taxonomic difficulty and the addition of a tenth genus to the nine recognized by Dawes (1941) as members of the family Aspidogastridae. Comparison of *T. elegans* as depicted by Jägerskiöld (1899) and Brinkmann (1957) with the descriptions of *M. cristata* by Faust and Tang (1936), Dollfus (1958), and the present specimens, discloses differences in host and geographic distribution, in size and form, in manner of growth (the proliferative zone is pretesticular in *T. elegans* and posttesticular in *M. cristata*), in extent of uterus, and in location of the gonads, especially the testis. These differences appear adequate to distinguish between two generic concepts.

Three immature specimens of *Macraspis*, probably *M. elegans*, were found by Manter (1931) in the intestine of a southern kingfish, *Menticirrhus americanus*, taken near Beaufort, North Carolina. From their location, the worms had apparently been ingested with food of the host, which according to Breder (1929) consists largely of invertebrates and small fishes, and which led Brinkmann (1957) to suggest that *M. americanus* may serve as a transmitting agent for *Macraspis*. Manter (1954) listed *M. elegans* from the gall-bladder of *Callorhynchus milii* taken in New Zealand waters and reported a second species of *Macraspis*, represented by worms taken previously from the gall-bladder of an undesignated "dogfish" by students at Victoria University College, Wellington, N. Z. Two specimens each measured 43 mm. in length, with about 300 acetabula, the testes near the middle of the body, the ovary about one-seventh of the body length from the anterior end, and the eggs measured 122 to 132 microns in length by 81 to 96 microns in width. Dollfus (1958) referred these specimens to *M. cristata*; also others from the gall-bladder of *Mustelus* (*Cynias*) *canis* (S. L. Mitchill) taken near Dakar, Senegal. The largest of these worms measured 113 mm. long, 2.5 mm. wide and 3.0 mm. thick. In a postscript, Dollfus reported receipt of numerous additional specimens found in the gall-bladders of *Scoliodon terrae-novae* and *Rhinobatus cemiculus*, taken near Gorée, Sénégal. In an earlier paper, Dollfus (1956) had divided the family Aspidogastridae into two subfamilies: Aspidogastrinae and Macraspidinae. Because of new information from the African material, Dollfus (1958) revised his diagnosis of the subfamily Macraspidinae, but the suppression of *Macraspis* as a homonym eliminates the name of the subfamily and, indeed, the division of the family serves no useful purpose.

The aspidogastrid trematodes have been the subject of controversy for more than a century. The type species, *Aspidogaster conchicola* von Baer, 1827, lives in the pericardial and renal cavities of fresh-water mussels in Europe, North America, and China, and has been reported from the digestive tracts of various fishes and turtles that presumably acquired the worms by eating infected mollusks. How long the worms can survive in such predator hosts is unknown, but Van Cleave and Williams (1943) recorded a period of fourteen days after introducing the worms into the stomach of a turtle, *Pseudemys troosti*. A number of other species have been described from molluscan, piscine and chelonian hosts, and were arranged in nine genera by Dawes (1941). All are members of the family Aspidogastridae Poche, 1907.

The first realistic classification of the trematodes was made by Burmeister (1856) who arranged them in three groups: (1) Pectobothrii (*pēktos,* compact, firm; *bothros,* pit) with firm, hard suckers; (2) Malacobothrii (*malakos,* soft) with soft, flexible suckers; and (3) Aspidobothrii (*aspis,* shield) with multiloculate adhesive organs. Van Beneden (1858) divided the Trematoda into two groups: "Monogeneses," those with a single sexual generation, and "Digeneses," those in which a sexual generation alternates with asexual generations. The names applied to the two groups were latinized by Carus (1863) as Monogenea and Digenea. Van Beneden made no attempt to allocate the Aspidobothrii, but early authors generally included the aspidogastrids in the Digenea, although it was known from the studies of Aubert (1855), Voeltzkow (1888) and others that *Aspidogaster conchicola* at least and perhaps others, did not have asexual generations in the life-cycle.

Development of knowledge concerning the life-cycle of the trematodes was complicated by erroneous postulates. After alternation of generations in certain lower invertebrates was established by Steenstrup (1842) and the discovery of the developmental cycle of *Fasciola hepatica* by Leuckart (1882) and Thomas (1883), alternation of generations in the Digenea appeared firmly established. However, Grobben (1879) and (1882), after demonstration of parthenogenesis in other invertebrates, concluded that the life-cycle of trematodes does not involve an alternation of sexual and asexual generations, but is heterogonic, the sexual generation alternating with parthenogenetic ones. This idea was adopted by Sinitsin (1911) who designated the generations in the molluscan host "Parthenita" in contrast to the sexual generation which he termed "Marita." Stunkard (1940, p. 6) stated, "So far as I am aware, parthenogenesis has never been established by critical and competent methods in either sporocysts or rediae, and the best recent work has shown that in several species it does not occur." The terminology has been espoused by many authors and still persists. Odening (1960) employed the terms although Schäller (1960), in the same number of the same journal showed that reproduction in the intermediate host is strictly asexual.

Although alternation of generations was widely accepted in the life-cycles of the Digenea, there were certain anomalies. The life-history of the holostomes appeared to be an exception, since no sporocyst or redial stages had been observed and the encysted tetracotyliform larvae developed sexual maturity in vertebrate hosts. In the first edition of his "Parasiten des Menschen," Leuckart (1863) voiced the suspicion that the embryo of the holostomes may develop directly into the tetracotyle. The larva which emerges from the egg was first designated a miracidium by Braun (1893). The idea was accepted by von Linstow (1877) who pointed out that such a method of development is distinct from monogenetic and digenetic cycles and appears to be intermediate between them. Leuckart (1889) adopted the theory of von Linstow and termed the development of the holostomes "Metastatic," *i.e.,* intermediate between monogenetic and digenetic. This concept was endorsed by Brandes (1890) and other authors who believed that the larvae long known as "Tetracotyle" and "Diplostomum" developed by metamorphosis of the miracidium without sporocyst or redial generations. It persisted until Lutz (1921), Mathias (1922) and Ruszkowski (1922), independently, showed that the tetracotyliform larvae develop from furcocercous cercariae and that the life-cycle is strictly digenetic. Actually, there is no intermediate condition between monogenetic and digenetic and the concept "Metastatica" of Leuckart was based on a false presumption.

Monticelli (1888) arranged the digenetic trematodes into four families and included *Aspidogaster* in the family Amphistomeae. Shortly thereafter Brandes (1890) divided the Diplostomeae of Monticelli into three families: Diplostomidae, Hemistomidae, and Holostomidae. The anomalous position of the aspidogastrids, and the acceptance of metastatic development of the tetracotyliform larvae, made the division of the trematodes into Monogenea and Digenea somewhat incongruous and artificial. Accordingly, Monticelli (1892) reverted to the scheme of Burmeister, and divided the order Trematoda into three suborders: Heterocotylea, Aspidocotylea, and Malacocotylea. Actually, the result was to divide the Digenea into the Aspidocotyles and Malacocotylea, since the Heterocotylea was equivalent to the Pectobothrii. Discussing the situation, Braun (1893) noted that the aspidogastrids are monogenetic but morphologically like the distomes, whereas *Gyrodactylus* is not monogenetic although it is morphologically like the polystomes. He stated (p. 889), "Es ist aber sehr fraglich, ob wir berechtigt sind, ein System der Trematoden ausschliesslich auf ihre verschiedene Entwicklungsweise zu grunden; abgesehen davon, dass dieselbe bei vielen Gattungen absolut unbekannt ist, demnach die Einfügung derselben in das System nach ganz anderen Gesichtspunkten vorgenommen werden muss, folgen wir sonst nirgends diesem Princip ausschliesslich und haben es bei den Monogenea resp. *Gyrodactylus* mit Recht nicht befolgt; ja wir stellen es ziemlich in den Hintergrund, da seine alleinige Anwendung bei der nicht selten recht verschiedenen Entwicklungsweise notorisch nahe verwandter Arten resp. Gattungen zu sehr sonderbaren Systemen führen müsste."

Jägerskiöld (1899) discussed the anomalous condition of the aspidogastrids and Odhner (1902) showed that in general morphology they agree substantially with the digenetic distomes. Stunkard (1917) reviewed the literature pertaining to the family, described *Cotylaspis cokeri* Barker and Parsons, 1914 from the intestine of *Malacoclemmys lesueurii*, and discussed the morphology of the group. Concerning its systematic position he stated (p. 82), "Whether the Aspidogastridae are primitive forms or secondarily degenerate is at yet undecided. The simple and archaic character of the intestine, the eye spots, the direct development and the ectoparasitic habit as it occurs in the family, together with the parasitic infection of mollusks by adult forms strongly suggests a very primitive and ancient group. It is probable that complete evidence concerning the structure and life-history of this family would go a long way toward solving the problem of whether the invertebrate or the vertebrate is the original host and the attendant problem of the origin of double hosts." In this connection, Leuckart (1879) compared *Archigetes*, a progenetic cestode which becomes sexually mature in tubificid annelids, with *Aspidogaster* and suggested that the aspidogastrids are essentially sexually mature rediae.

Studies by different authors on development of members of the family provide data of systematic value. Voeltzkow (1888) reported that as it emerges from the egg, the young *Aspidogaster conchicola* is a distome, with a simple posterior sucker. In the sucker, the septa which produce the multiloculate condition are developed internally and the organ is then everted to form the shield-shaped adhesive disk. Other accounts were made by Faust (1922) and Williams (1942). According to Faust, on emergence from the egg, the larva has paired clusters of cephalic glands whose ducts open just anterior to the oral sucker. He stated that these glands are analogous to the salivary glands of the redia of *Cercaria equitator* Sinitsin, 1911 and could lend support to the view that the aspidogastrid is a redia. Further data were provided when Odhner (1898) showed that *Stichocotyle nephropis* Cunningham, 1884, which was described from larvae encysted on the intestinal wall of *Nephrops norvegicus*, becomes adult in the bile ducts of the liver of rays, *Raja clavata*. This discovery showed that *S. nephropis* has at least two hosts in the life-cycle, but the manner in which the lobster becomes infected is yet undisclosed. Nickerson (1895) reported the species encysted on the intestine of the American lobster, *Homarus americanus*. *Cotylaspis insignias* Leidy, 1856, is a parasite of various unionid species in North America. Stunkard (1917) reported it from *Unio pustulosis, Lampsilis gracilis,* and four species of *Anodonta*. Osborn (1904) gave an account of the distribution, habits, and anatomy of the species and described a young individual which had a simple ventral sucker, no eye-spots, and two entirely distinct and separate excretory systems and pores. This condition of the excretory system is identical with that in rediae and very young cercariae of the Digenea and according to Osborn supports the idea proposed by Leuckart that the aspidogastrids are sexually mature rediae. Wharton (1939) reported specimens of *Lophotaspis vallei* (Stossich, 1899) from the stomach of a large loggerhead turtle, *Caretta caretta*, taken in Gulf County, Florida. Young specimens of the same species, described as nymphs, were found in the conch, *Fasciolaria gigas,* from the same area. These mollusks are eaten by the turtle. According to Wharton, the emerged larva has eye-spots, oral and posterior suckers, three patches of cilia, and can both crawl and swim. Accordingly, the larvae can enter the mantle cavity of gastropods and develop into juveniles. Wharton noted that *Lophotaspis macdonaldi* Monticelli, 1891 is an immature individual from the Australian marine gastropod, *Melo* sp. Brinkmann (1957) reported on the development of *Macraspis elegans*. The eggs are embryonated in the uterus; the larvae on emergence lack cilia, have poorly developed anterior and well developed posterior suckers. He found a series of stages from very small specimens to fully mature ones in the gall-bladder of *Chimaera monstrosa* and concluded that *M. elegans* has a direct development without alternation of generations. However, he stated that the species may have an intermediary or transport host. This is indeed very probable; otherwise it is difficult to see how the emerged larvae could reach the final host.

Present knowledge shows the aspidogastrids to be primarily parasites of mollusks, but able to survive for considerable periods of time in predator hosts, *e.g.;* species of *Aspidogaster, Cotylogaster* and *Lophotaspis* in fishes and turtles. Moreover, certain of them, *Stichocotyle nephropis, Multicalyx cristata,* and *Taeniocotyle elegans,* have added intermediate, secondary or transport hosts in the life-cycle, although so far as known alternation of the sexual generation with an asexual one does not occur. The larvae emerge from the eggs as small distomes and often retain features characteristic of the rediae of digenetic trematodes, but are quite distinct morphologically from the larvae of the Monogenea which are limited to aquatic vertebrate hosts. Stunkard (1946) affirmed that the affinities of the aspidogastrids are clearly with the Digenea, although present information is insufficient to determine whether their life-cycle is primitive, or secondarily simplified. They constitute an aberrant, isolated group of parasites of mollusks and lower vertebrates which feed on such mollusks, and infect both marine and fresh-water hosts in all parts of the world. Members of the family form a homogeneous, coherent systematic group. *Stichocotyle* and *Taeniocotyle* agree in possessing a single row of alveoli and in this respect they differ from all other genera, whose members have three or four rows of alveoli. But *Taeniocotyle* has a single testis, a feature which it shares with *Aspidogaster, Cotylaspis, Lissemysia, Lophotaspis* and *Lobatostoma,* while *Stichocotyle, Cotylogaster* and *Multicotyle* have two testes. Many common features are shared and subdivision of the family is not justifiable.

As noted earlier, in his classification of 1892, Monticelli virtually divided the Digenea of van Beneden into the Aspidocotylea and Malacocotylea. It is evident that the aspidogastrids do not have a digenetic life-cycle and must be excluded from the Digenea. Accordingly, different authors have proposed to place them in a category intermediate between Monogenea and Digenea, but there is no intermediate condition. Either alternation of sexual and asexual generations does or does not occur in the life-cycle. Since the categories of Monticelli are merely rechristenings of the earlier ones of Burmeister, I propose to restore the original groups and arrange them in accord with morphological and developmental data. To this end, the Aspidogastridae and Digenea are included in a higher taxonomic unit, the subclass Malacobothridia Burmeister, 1856. The classification of the Pectobothridia appears generally acceptable but the arrangement of the Digenea is disputed by La Rue (1957) and Odening (1960). An incomplete system may be sketched as follows:

Class Trematoda

Subclass Pectobothridia Burmeister, 1856.
Firm, hard suckers, generally ectoparasitic on aquatic vertebrates and monogenetic; exceptions, *Gyrodactylus* and the polystomes of amphibians; one host.
Order Monopisthocotylea Odhner, 1912.
Suborder Gyrodactyloidea Johnston and Tiegs, 1922.

Suborder Capsaloidea Price, 1936.

Order Polyopisthocotylea Odhner, 1912.
Suborder Polystomatoidea Price, 1936.
Suborder Diclidophoroidea Price, 1936.

Subclass Malacobothridia Burmeister, 1856.
Soft, flexible suckers; generally endoparasitic in invertebrates and vertebrates; begin life-cycles in mollusks; with 1, 2, 3 or 4 hosts.

Order Aspidobothrea Burmeister, 1856.
Single family, Aspidogastridae Poche, 1907.
Order Digenea van Beneden, 1858.
Suborder Strigeatoidea Railliet, 1919.
(= Order Strigeatoidea La Rue, 1926).
Suborder Echinostomatoidea Faust, 1929.
(= Order Echinostomida La Rue, 1957).
Suborder Plagiorchioidea Dollfus, 1930.
(= Order Plagiorchiata La Rue, 1957).
Suborder Opisthorchioidea Faust, 1929.
(= Order Opisthorchiata La Rue, 1957).

Members of the Malacobothridia, like those of the Cestoda, begin their life-cycles as parasites of invertebrates, the former principally in mollusks, the latter in arthropods. Both groups are undoubtedly of great geologic age and there is evidence that present families have evolved together with their hosts (Stunkard, 1957). The cestodes are more degenerate or more highly specialized and more host-specific than the trematodes, and presumably have a longer parasitic history. Both groups are quite distinct from the Pectobothridia which have a distinctly different structure, different life-cycles, and presumably a different phylogenetic history.

It appears that the aspidogastrids and the digenetic forms have descended from a common turbellarian-like ancestor which initially was parasitic in mollusks; that the aspidogastrids never acquired asexual reproduction and become mature in the molluscan hosts or in vertebrates which feed on such hosts, whereas members of the Digenea developed polyembryonic asexual reproduction in the mollusk and with the acquisition of vertebrate hosts, sexual maturity was more and more deferred to worms in the definitive hosts. Acquisition of the longer-lived, wider-ranging vertebrate hosts facilitated dispersal and prolonged the life of the parasites, thus increasing reproductive capacity and survival value of the species. The frequent appearance of progenesis and the demonstration (Stunkard 1959, 1960) that the life-cycle of *Asymphylodora amnicolae* can be completed in the snail without the intermediation of the usual vertebrate host, lend support to the thesis that originally the Digenea were parasites of mollusks and that secondarily they acquired vertebrate hosts.

SUMMARY

Taeniocotyle nom. nov. is proposed to replace *Macraspis* Olsson, 1869, homonym of *Macraspis* MacLeay, 1819, a coleopterous insect. It designates certain trematode worms, from the gall-bladders of selachian fishes, that belong to the family Aspidogastridae Poche, 1907 and the order Aspidobothrea Burmeister, 1856. The systematic position of this group, often regarded as intermediate between the Monogenea and Digenea, is reviewed. From morphological and developmental evidence, the Aspidobothrea and Digenea are included in the subclass Malacobothridia Burmeister, 1856.

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THE POLYCHAETE CERATONEREIS TRIDENTATA AS A PEST OF THE SCALLOP AEQUIPECTEN GIBBUS

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Because of the importance of clams, scallops, mussels, and oysters as a source of food, the biology of bivalve mollusks has received much attention. Much ecological research has centered on the associates of these bivalves, in recognition of the possibility that they may destroy the bivalves, or restrict their growth or reproduction, and, in this manner, may compete with man's fullest utilization of the bivalve populations. These potentially detrimental associates include predators that feed on the mollusk, fouling organisms that attach to the shell, commensals and ectoparasites that live between the shells, invaders that live in the shell itself, and endoparasites that live within the tissues and organs of the bivalve.

The diverse organisms that can be termed "invaders" of the shell usually receive little consideration until, under optimal environmental conditions, a population "bloom" of the invader damages the shells sufficiently to cause mortalities or interfere with the processing or marketing of the shellfish. Outstanding examples of organisms that invade the shells of oysters are boring sponges (*Cliona* species), spionid polychaetes (*Polydora* species), and a fungus (Korringa, 1952). Usually the relation of such species to their host is quite distinct from that of the commensal or parasitic organisms, such as pinnotherid crabs, that live between the bivalve's shells. The species that invade the shell often have a detrimental effect upon their host, and to this extent, may consequently be considered parasites.

Since 1958, the calico scallop has been the object of a small commercial fishery on the North Carolina coast; however, the biology of this species has received little attention. Inasmuch as they can affect the population dynamics of the scallop, the enemies of this species, including its parasites, are important to this fishery.

The spionid polychaete, *Polydora websteri* Hartman, has long been considered a pest of bivalves. It penetrates the calcareous shells of oysters, scallops, and mussels, where its presence may stimulate the mollusk to secrete extra layers of shell around the worm's burrow. In this manner, *Polydora* causes its host to divert energy to shell deposition, detracting from its host's condition and suitability for market, and perhaps leaving its weakened host prey to other enemies and diseases (Lunz, 1940; Mackin and Cautheron, 1952; Medcof, 1946; Owen, 1957; and others). The typical deposits of shell around *Polydora websteri* are termed "mud blisters" because of their characteristic dark color and mud inclusions.

In January, 1959, large brown blisters were found in the upper valves of two calico scallops (*Aequipecten gibbus*), of a group of 20 which had been dredged off Ocracoke Island, North Carolina. The observed blisters contrasted with mud blisters produced in this bivalve in response to *Polydora websteri*, which is common in this area (Hartman, 1945). Whereas *Polydora* blisters were typically 5 to 15 mm. in greatest length in the scallops, these blisters were larger, 20 and 25 mm. in length. The interior of one blister communicated with the mantle cavity of the scallop through a hole in the scallop's mantle. Both blisters harbored an annelid identified as *Ceratonereis tridentata* (Webster, 1879) (Polychaeta: Nereidae), a species widely distributed along the Atlantic seaboard. Because such a relationship to mollusks had not been reported for this annelid species, a more extensive study was undertaken to determine the prevalence of such an infestation and its effects on scallops.

DRY SHELLS

Shell mounds outside a scallop shucking house at Salterpath, N. C., provided an easily accessible source of fresh shells for examination. These shells had been dredged commercially in March, 1961, off Core Bank, south of Ocracoke, N. C., near the source of the first infested scallops. Hundreds of shells bore blisters apparently caused by *Ceratonereis*. In an effort to obtain a quantitative sample, 600 valves were examined for the presence of blisters. Only upper valves were used in order to avoid counting the same scallop twice. Recognition of *Ceratonereis* blisters was made difficult by an intense infestation of *Polydora* blisters. Because it was often difficult to distinguish between a marginal *Ceratonereis* blister and a series of contiguous *Polydora* blisters, those of questionable origin were not counted. *Ceratonereis* blisters were recognized in 81 of 600 shells examined (16%); *Polydora* blisters were noted for 116 of 300 shells (39%). The incidence of infestation approximated the level indicated by the earlier observations of *Ceratonereis* in *Aequipecten gibbus;* coincidentally, the *Polydora* infestation appeared to be much more intense.

TABLE I

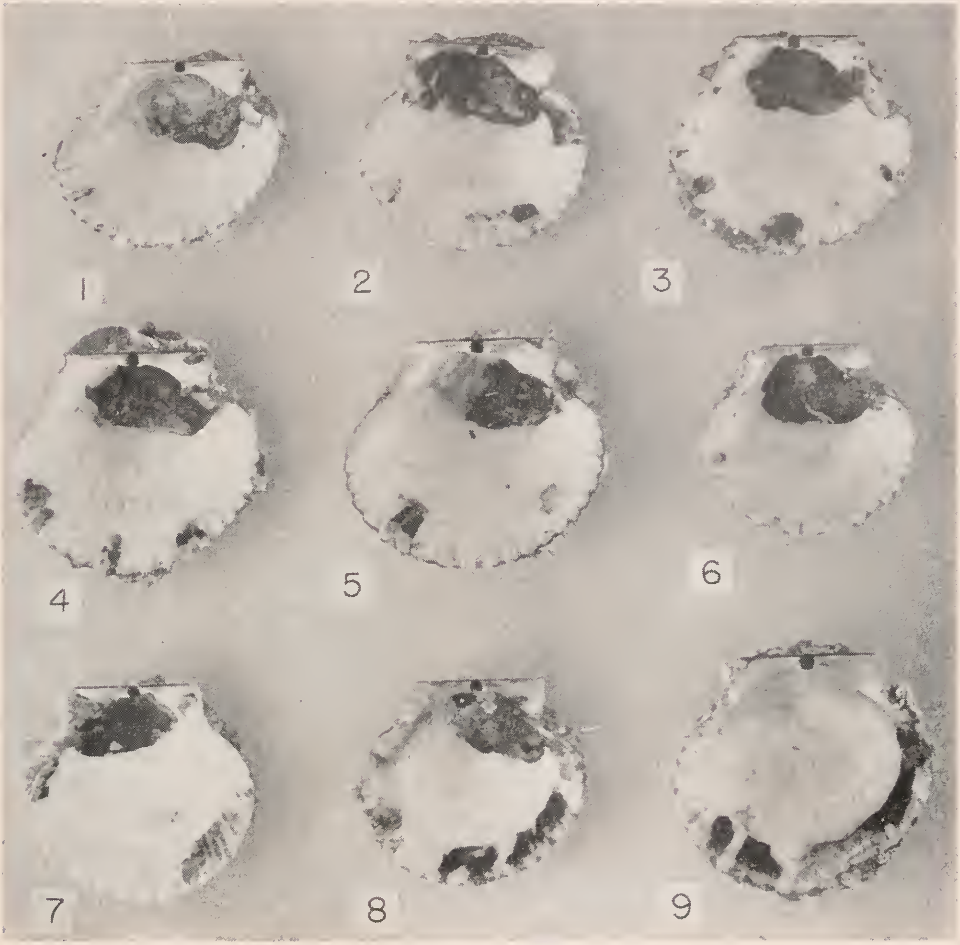
Distribution of Ceratonereis blisters in Aequipecten gibbus

Position	Umbonal	Marginal	Totals
Upper valve	58	27	85
Lower valve	15	14	29
Totals	73	41	114

INFECTION OF LIVING SCALLOPS

Fresh scallops dredged April 3, 1961, off Core Bank, were examined for the presence of blisters caused by *Ceratonereis tridentata*. As each specimen was opened, observations were made of possible damage to soft parts. Wherever there was any question as to the identity of the causative agent, every effort was made to locate and identify the inhabitant of the blister. *Ceratonereis* blisters were found in 118 of 486 scallops (24.3%). Of these, 10 (2%) contained double *Ceratonereis* infestations. Careful examination revealed that about 99% of the scallops were infested by *Polydora websteri* burrows and resulting blisters.

The discrepancy between these infestation levels and those obtained from dry shells may be attributed to possible destruction of blisters in the commercial shucking process and to the omission of infections in lower valves among the dry shells. *Ceratonereis* blisters occur in the lower (right) valve about one-third as often as in the upper (left) valve (Table I), and when a corresponding correction is applied to the original level of incidence, there is satisfactory agreement.

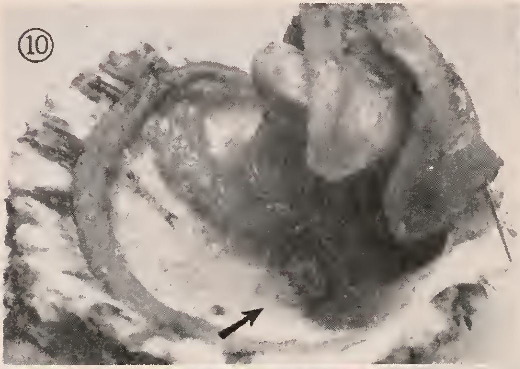


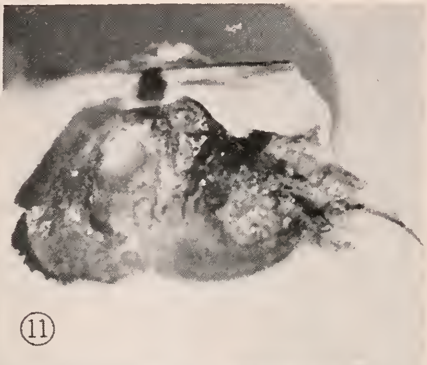
FIGURES 1–9. Interior views of *Aequipecten gibbus* shells showing blisters associated with *Ceratonereis tridentata*. Figures 1–8 show umbonal blisters; Figure 9 shows a marginal *Ceratonereis* blister. Figures 3–5, 8–9 also show smaller marginal blisters caused by *Polydora websteri*.

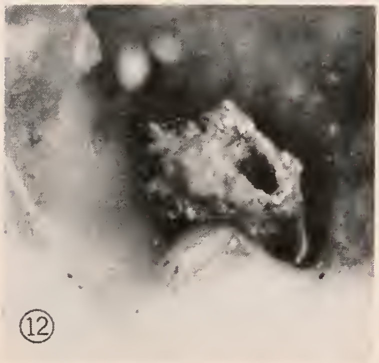
NATURE OF BLISTERS

The interior of the valves of *Aequipecten gibbus* is typically composed of a smooth, pearly-white layer of calcium carbonate crossed by radiating shallow grooves that correspond to the radial ribs of the exterior sculpture. In many shells faint brown stains may be suffused through the shell layers adjacent to the dorsal wings (ears) and on the hinge line.

Blisters caused by *Ceratonereis* are composed of thin, dark brown layers of organic matrix (conchiolin) that contrast noticeably with the white interior of the shell (Figs. 1–9). They are not easily confused with the brownish suffusion near the hinge nor with the red pigment of the exterior shell layers. Where the blister meets the margin, additional layers of conchiolin reinforce the edge. A similar thickening may be present around a perforation of the blister wall. At this stage, the conchiolin blister is still relatively flexible, although it becomes hard and brittle if dried. In later stages, white droplets of nacreous shell material are scattered over the dark foundation produced by successive conchiolin layers.







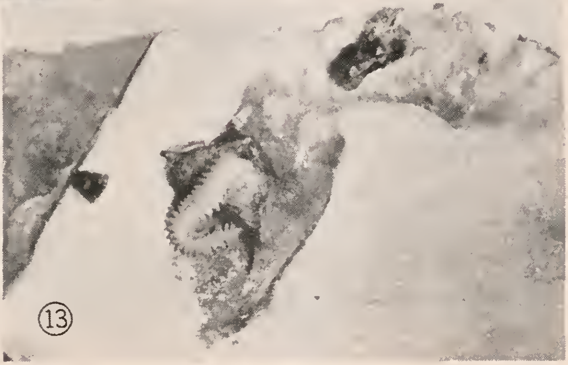


FIGURE 10. View into the mantle cavity of preserved scallop showing anterior end of *Ceratonereis tridentata* and mud tube projecting from mud-blister. Right valve removed and right mantle pulled aside.

FIGURE 11. Enlarged view of umbonal *Ceratonereis* blister showing natural perforation, and extension to anterior edge of shell.

FIGURE 12. Enlarged view of anterior end of *Ceratonereis tridentata* and mud tube projecting into mantle cavity through perforation in mantle and blister wall.

FIGURE 13. Umbonal blister with posterior end of *Ceratonereis tridentata* projecting from natural perforation. Exterior opening and blister development show clearly on anterior margin (to right).

This nacreous material confers hardness to the blister wall, producing a thin crust around the invader, and as nacreous deposits accumulate, the dark color of the underlying blister becomes obscured. Where the nacreous crust has been broken, there are signs of repair involving both conchiolin and additional nacreous deposits. If the mantle has been prevented from returning to its normal position at the margin, a narrow rift may remain between the new and old layers of shell.

Generally, blisters caused by *Ceratonereis* are oval or crescent-shaped, depending on their position in the scallop shell. They may elevate the inner shell surface up to 4 mm., although most are only 2 or 3 mm. thick at the highest point. While the over-all surface area may vary considerably, from 1.3 to 8.5 cm.^{2}, most blisters occupy 3.5 to 4.0 cm.^{2} of the interior surface. In other terms, the average blister occupies approximately 12% of the available surface of the affected shell. In extreme cases and where two infections occur in the same shell, up to 25% of its surface may be covered by *Ceratonereis* blisters. A more or less round perforation can be found on the central wall of many blisters (Fig. 11), its edges thickened as though extra layers of conchiolin have been deposited at this point. This perforation is usually about 1 to 1.5 mm. in diameter. Adhering to the shell within a blister is a thin tube usually occupied by the polychaete. The tube is composed of fine sand grains loosely held together by mucus secreted by the worm. Usually, this tube is U-shaped, but in some cases, one end extends through the perforation beyond the edge of the blister (Figs. 10, 12). Where a functional opening to the exterior occurs at the shell margin, the channel may be as small as 0.3 mm. in diameter or as large as 2.5 by 1.5 mm. in cross-section. It is often somewhat hidden between subsequent deposits and the old shell layer. Usually, however, there is no external manifestation on the scallop shell of its internal deformation by *Ceratonereis*.

TABLE II

Distribution of marginal openings in umbonal blisters

Position	Anterior opening	Anterior and posterior openings	Posterior opening
Upper valve	109	24	2
Lower valve	16	4	0
Total	125	28	2
%	81	18	1

A series of *Ceratonereis* blisters in the calico scallop are shown in Figures 1–9. These blisters fall into two principal types on the basis of their shape and position in the scallop shell. The more abundant type (Figs. 1–8) is umbonal in position, *i.e.*, located under the umbo, dorsal to the adductor muscle or "heart" of the scallop; the other (Fig. 9) is marginal in position, located outside the pallial line (where the retractor muscles of the mantle attach to the shell). Marginal blisters are generally narrow and arched or crescentic, following the curvature of the margin, while umbonal blisters are more or less oval, usually with one or more extensions to the margin near the wings. Approximately 65% of the blisters caused by *Ceratonereis* occur in the umbonal position (Table I). As noted above, about 75% occur in the left valve, which is the upper valve in the normal orientation of the scallop. Most umbonal blisters have an arm that extends to the anterior margin immediately adjacent to the byssal notch, where the anterior wing joins the anterior shell border (Fig. 13). About 20% of umbonal blisters also possess a posterior extension across the posterior wing (Table II). Presumably both served at one time as a passageway for the annelid, but such channels to the exterior may have been closed by subsequent shell deposits. Independently of any marginal outlet, many blisters communicate with the scallop's mantle cavity through a perforation of the mantle and the conchiolin layer (Figs. 10–13).

Annelid Agent

Specimens of the annelid *Ceratonereis tridentata* were found in most blisters, sometimes with the anterior parts projecting into the mantle cavity from a hole in the blister (Fig. 12). Occasionally, when the host scallop had died before it was opened for examination, the blister was empty and a *Ceratonereis* was sometimes found crawling about on the exterior of the shell. Specimens of *C. tridentata* also were found frequently among the epifauna of the scallop shells, without apparent relation to the presence or absence of internal blisters. Several other species of nereid polychaetes may occur in this epifauna, from which this invasive species should be distinguished.

Ceratonereis tridentata has been adequately described by Hartman (1945, 1951), who has reported it as occurring in shelly bottoms from New Jersey to Texas. Specimens of *C. tridentata* recovered from blisters in the calico scallop measured 20 to 50 mm. in length and 1 to 2.5 mm. in width.

Effect on Scallop

Without doubt, the observed *Ceratonereis*-associated blisters were formed by the scallop's mantle in response to its irritation by this annelid. In addition to its deformation of the shell, this annelid clearly affects the soft parts of the host scallop. To a minor degree, the natural symmetry of the scallop tissues may have been distorted by the volume of the *Ceratonereis* blister, but a greater distortion may occur as a result of direct contact of the annelid with the tissues. The blister cavity typically communicates with the scallop mantle cavity through a perforation of the mantle. In the more abundant umbonal blisters, this mantle perforation occurs a short distance anterior and dorsal to the adductor muscle. The edges of these perforations are smooth and thickened, rather than torn and ragged as would be expected if they had been the result of accidental damage or a single penetration by the annelid. More important, the gills were often reduced on the infected side when the opening to the blister lay close to the gill region. In an extreme case, only about one-fifth of the gill tissue remained on the affected side. This condition appears to be either the result of physical erosion of gill tissue by the movements of the annelid among the gills, or the result of actual ingestion of gill tissue by *Ceratonereis*. Since the activities of *Ceratonereis* have not been observed in living scallops, the cause of this damage cannot be fixed with certainty. A more obvious malformation of infested scallops was a marked reduction in the central visceral mass. The bright red and cream-colored gonads that usually comprise the greatest portion of the visceral mass were obviously atrophied in certain specimens. In extreme cases, the volume occupied by this mass was only 25 to 30% of its volume in normal, uninfested scallops of the same shell dimensions. These deleterious effects upon tissues were more frequently observed in scallops with umbonal blisters than in those with marginal blisters. Undoubtedly, the secretion of extra shell layers in response to irritation by *Ceratonereis* requires energy that might otherwise be utilized in the growth or reproduction of the scallop. Where the gills are damaged, the scallop's intake of food would also be impaired, with a consequent additional decline in the scallop's condition. The observed atrophy of gonads probably reflects the degree to which extra shell deposition and gill damage affect the scallop's over-all condition.

Mode of Infection

Unlike many bivalve mollusks, scallops cannot close their shells perfectly, so that a gap remains at both ends of the ligament. Water passes through these slits in the normal swimming activity of scallops (Gutsell, 1930). At the anterior end, this gap coincides with the byssal notch, a deep indentation of the margin that is more developed in the right valve. Very young scallops of other species (*Aequipecten irradians, Placopecten magellanicus*) attach to objects by byssal threads that pass through such an opening (Gutsell, 1930; Merrill, 1961). Presumably, young calico scallops also attach by a byssus through this notch. In this species, these narrow slits provide an unprotected natural channel for invasion by *Ceratonereis.* In the formation of an umbonal blister, *Ceratonereis* probably makes its entry through these openings, inserting itself between the mantle and the shell, and then constructs its U-shaped tube with both ends at or near the shell margin. It may perforate the mantle under the umbo, establishing an opening into the mantle cavity. First the conchiolin layer, then successive nacreous layers are deposited over the affected areas. Once the second opening is established, the scallop's deposition of shell material may close off the original entry without adversely affecting the annelid. Such a sequence seems a logical course for the formation of umbonal blisters, and fits the observed stages.

In the formation of marginal blisters, a similar sequence of events must occur, as a result of penetration by *Ceratonereis* between the mantle edge and shell in lateral and ventral regions. There, the attachment of mantle to shell along the pallial line apparently restricts the penetration of the annelid to a marginal zone and consequently delimits blister formation. As an additional possibility, *Ceratonereis* may be a secondary invader in marginal blisters initially caused by some other agent, although only two cases were recognized in which this sequence might have occurred. In shells of the sea scallop, *Placopecten magellanicus,* Merrill (1960) found marginal deformations which he attributed to mud and other foreign material introduced under the mantle edge. Various sessile organisms which occupied the resulting blisters were not considered to have initiated their formation. Judged by the success of *Ceratonereis* in penetrating between the shell and mantle in the dorsal regions, this annelid would be well adapted to exploit any physical injury to the ventral margin of the calico scallop. However, while *Ceratonereis* may be in some instances a secondary invader in marginal blisters.

there is no indication that *Ceratonereis* is ever a secondary invader in umbonal blisters.

Discussion

A number of agencies, both living and non-living, may cause withdrawal of a bivalve's mantle and the subsequent production of blisters around the site of irritation. As Merrill (1960) found in the sea scallop, non-living matter, accidentally introduced between the shells of a bivalve, may stimulate the secretion of a marginal blister, which isolates this material from the bivalve's soft parts. Merrill (1960) considered the introduction of mud and debris between the sea scallop's valves to be a result of nearby dredging operations. Considerable quantities of sand have been observed in freshly dredged calico scallops, in which the mantle edge had been forced away from the shell margin. If such a scallop were unable to clean itself, or if the mantle edge had been damaged, subsequent deposition of shell materials would produce a blister somewhat resembling the marginal blisters produced in *Placopecten magellanicus.* Some marginal blisters may be produced in *Aequipecten gibbus* by this mechanism, for scallops with damaged margins and several with a deposit of coarse sand enveloped by a conchiolin blister were observed, without trace of any biological agent responsible for the blister malformation.

The steps of blister formation are essentially the same in these cases of blisters caused by non-living materials as they are in biologically-caused blisters. In both cases, the initial reaction is the deposition of many thin conchiolin layers, followed by successive layers of white nacreous material. In time, the irregularity in the shell may be obscured by further nacreous deposits.

Probably the best-known animal that causes blister formation in bivalve mollusks is *Polydora websteri* Hartman, a well known pest of oysters which also occurs in many other bivalves, including the calico scallop. It can be distinguished with ease from *Ceratonereis tridentata,* for these two polychaetes belong in very different families. In contrast with *Ceratonereis tridentata, Polydora websteri* is usually slender and small, 20 mm. or less in length (Hartman, 1951). In calico scallops, *Polydora websteri* builds small, U-shaped tubes at the margin between the shell and mantle, where it accumulates mud and silt around its burrow (as in Figs. 3–5. 8). In time, layers of conchiolin wall off this accumulated material and the worm, and thus form a small, dark marginal blister, which averages about 7.5 by 9.0 mm. in these scallops. Some *Polydora* may tunnel obliquely through the shell, producing an often-twisted, U-shaped gallery up to 0.5 mm. in diameter. If such a gallery perforates the inner shell surface, the scallop may secrete resistant layers of conchiolin over the site of perforation. Many stages of *Polydora* infestation in *Aequipecten gibbus* have been recognized and observed, including some blisters formed over umbonal perforations, some of which had been covered and almost obliterated by subsequent nacreous deposits.

Interior blisters may be formed in reaction to several other living agents. Sponges of the family Clionidae characteristically inhabit calcareous materials, including the shells of living mollusks, often creating extensive systems of galleries and pores (Old, 1941; Hartman, 1958). Upon penetration to the inner shell surface of a living oyster, a boring sponge stimulates the secretion of extra shell layers, which form a yellowish or greenish blister over the penetration. In addition, pyramidellid gastropods of the genus *Odostomia* may cause marginal pockets similar to *Ceratonereis* blisters. By irritating the oyster's mantle in the course of feeding on the European oyster, *Odostomia eulimoides* causes the oyster to deposit shell layers around a small marginal pocket (Cole and Hancock, 1955). This ectoparasitic snail may then move into the resulting pocket, and feed upon the oyster's mantle from that position. In a similar fashion, the feeding of *O. scalaris* on the blue mussel may stimulate a similar deformation of the mussel shell.

These various blister-producing organisms derive different benefits from the association with their bivalve hosts. Like those on the outside of the shell, fouling organisms found within the shells of sea scallops primarily receive a secure attachment from their host. Additionally, they may receive a degree of physical protection from their predators. In a similar way, *Polydora* and boring sponges obtain protection from the shells they inhabit. In addition to protection from predators, the pyramidellid gastropods receive nourishment from their hosts, and may be considered as parasites. In view of its damage to the shell and its effects on the tissues of its host, *Ceratonereis tridentata* may be considered a parasite also. While it benefits from its association with a scallop, it may produce deleterious morphological and, probably, physiological changes in its host. However, because *Ceratonereis* commonly occurs in another habitat, living independently of the scallop, it should be considered as only a facultative parasite of the calico scallop.

No account of the food or feeding habits of *Ceratonereis tridentata* has been published. *Ceratonereis* specimens living in umbonal blisters with openings to the mantle cavity may receive some nourishment directly or indirectly from the scallop (*i.e.*, from the scallop's tissues or from its food). Although the erosion of the gills could be the result of their ingestion by *Ceratonereis*, it may be the result of physical erosion caused by the worm's presence in or passage through the gill region. The observed condition resembles the erosion produced in gills of the American oyster, *Crassostrea virginica*, by the presence of the oyster crab, *Pinnotheres ostreum*. The oyster crab ingests food filtered from the water by the oyster's gills, and in the process, its appendages damage the gills (Stauber, 1945). In a similar fashion, *Ceratonereis* may gather planktonic food from the scallop's gills, food which had been filtered and formed into mucous strands in transit to the scallop's mouth. Outside the scallop, a similar diet might be obtained from a plankton-feeder among the epifauna of the shell. Only laboratory observations of *Ceratonereis* could provide definitive information on its feeding habits.

Ceratonereis tridentata is indeed a pest of the calico scallop, with a 24% infestation, a relatively high level of incidence. It is interesting to compare the proportion of double infestations (2%) with the over-all level of infestation. By the law of probability, if the occurrence of a second infestation were independent of the presence in the scallop of a preceding *Ceratonereis* infestation, the incidence of double infestations would be about 5% (*i.e.*, the square of the incidence of single infestations). The observed incidence of double infestations is considerably lower than the theoretical level. Calico scallops being rather small, a typical *Ceratonereis* blister occupies a major part of the shell area (about 12%), and apparently may repel a second *Ceratonereis* infestation.

Because only the adductor muscle ("heart") of a scallop is utilized for food in this country, the presence of a *Ceratonereis* blister in its shells does not affect the preparation of a scallop for market. The principal effect of *Ceratonereis* is to reduce the growth and reproductive potential of the calico scallop. In this manner, *Ceratonereis* infestations may detract from the ability of the survivors of each fishing season to effect repopulation.

Undoubtedly, the superficial resemblance between *Ceratonereis* blisters and those caused by *Polydora* has delayed the recognition of *Ceratonereis* as a pest of scallops. Nevertheless, the incidence of *Ceratonereis* blisters has probably increased since 1958 in the local scallop population, as a result of the fishing operation itself. By introducing sand between valves and through injuries to mantle margins, the operation of dredging rigs probably leaves many of the surviving scallops more susceptible to *Ceratonereis* invasion. Concurrently, a similar increase has occurred since 1958 in the incidence of *Polydora* blisters in this scallop population.

Other evidence indicates that *Ceratonereis* may invade the shells of other scallops. Essentially similar blisters in the umbonal region have been found in shells of the bay scallop, *Aequipecten irradians*, and in one shell of the sea scallop, *Placopecten magellanicus*. In both cases, however, these were dry shells and no specimens of *Ceratonereis* were recovered. The incidence of such blisters and the identity of the causative agent should be investigated for these two species.

These specimens were obtained and studied during the course of research supported by grants (G-5838 and G-13952) from the National Science Foundation to Dr. I. E. Gray of Duke University, and aided by the Cape Hatteras National Seashore of the National Park Service.

SUMMARY

The nereid polychaete *Ceratonereis tridentata* has been found occupying mud-blisters in the calico scallop, *Aequipecten gibbus,* from off North Carolina. Characteristic *Ceratonereis* blisters were found in 24% of live scallops examined. These blisters can be distinguished easily from those of *Polydora websteri* by their larger size and often by their location in the scallop shell. Blister cavities may communicate with the mantle cavity or with the exterior. Anomalous malformations of the gills and the gonadal mass often accompany blisters having communication with the scallop mantle cavity. *Ceratonereis tridentata* is a facultative parasite of this scallop, also occurring among the epifauna of its shell.

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ON THE BIOLOGY OF THE MESOGASTROPOD TRICHOTROPIS CANCELLATA HINDS, A BENTHIC INDICATOR SPECIES

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The observations on the mesogastropod, *Trichotropis cancellata* Hinds, with which the paper is concerned, were made during a period of some ten weeks in the summer of 1959 spent at the Friday Harbor Laboratory of the University of Washington, Seattle. They were supplemented by examination of further samples of living animals later that year in Seattle and during the early months of 1960 when working at the Hopkins Marine Station, Pacific Grove.

This research was not premeditated. It arose out of interest in an animal never previously encountered by the author. Thus, while a variety of most interesting points regarding ciliary feeding, protandric hermaphroditism, adaptation to a restricted range of bottom conditions and the mode of evolution of mesogastropod limpets were disclosed, no attempt could be made to probe deeper than was possible by examination of the living animal, although a few points have been confirmed and conclusions strengthened by subsequent sectioning of fixed material. But it is hoped that this general study may stimulate more detailed work. Particularly desirable would be a histological and experimental study of the protandric reproductive system over the two-year life span postulated in this paper. More precise information, to be obtained by means of grab samples, about the precise distribution of this species in relation to bottom substrates could also be most informing.

It is a pleasure to record thanks to colleagues of the summer season of 1959 at Friday Harbor, especially to Dr. Dixy Lee Ray and to Dr. R. L. Fernald who also sent additional samples of *Trichotropis* to Pacific Grove during February and March, 1960. Mr. Jefferson Gonor kindly reported on animals left in aquarium tanks at Friday Harbor and sent samples of these animals. Dr. J. Connell and Mr. G. Bakus identified species of barnacles and sponges which live on the shells of *Trichotropis*. At Pacific Grove every facility needed was supplied by the Director, Dr. L. R. Blinks, and members of his staff. The figures which illustrate this paper have been prepared by the author's research assistant, Miss J. I. Campbell. Grateful acknowledgment is also made to the United States Educational Commission in the United Kingdom for award of a Fulbright Travel Grant and to the Carnegie Trust for the Universities of Scotland for a grant towards expenses while working at Pacific Grove.

SYSTEMATIC POSITION

The genus *Trichotropis* Broderip and Sowerby 1829 is one of 10 genera in the Family Trichotropidae which, with the Capulidae and the Calyptraeidae, constitutes the Superfamily Calyptraeacea (Thiele, 1931). But, as will later be shown, relationship to the Capulidae is certainly much closer than to the Calyptraeidae. Species of *Trichotropis* possess a thin shell covered by a relatively very thick periostracum which is thrown into the characteristic rows of long bristles (Fig. 1) which are responsible for the common name of these hairy-shells. This periostracum soon wears away apically when the underlying calcareous layer speedily becomes eroded.

Abbott (1954) in his account of American Mollusca lists four species from northern seas: *T. borealis* Broderip & Sowerby which ranges from the Arctic to British Columbia and to Maine (also to northern Europe), *T. cancellata* Hinds from the Bering Sea to Oregon, *T. bicarinata* Sowerby from the Arctic to Alaska and to Newfoundland, and *T. insignis* Middendorff from Alaska to northern Japan. Thus, while all occur in the North Pacific only two species also inhabit the Atlantic.

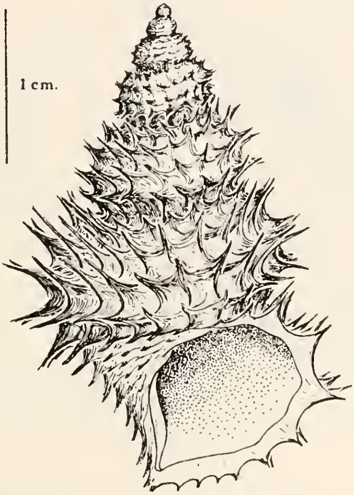

FIGURE 1. *Trichotropis cancellata*, empty shell showing characteristic spiral rows of periostracal spines.

SHELL AND INTACT ANIMAL

Shell

Both Oldroyd (1924), who quotes the description of Hinds from the zoology of the voyage of H.M.S. *Sulphur,* and Abbott (1954) give an account of the shell of *T. cancellata.* It is that of a typical mesogastropod with height about double the basal diameter, and with a well pointed spire. The deeply separated whorls each bear four or five spirally running keels across which run the rows of axial ribs which give the characteristic cancellated appearance. The shell attains greater lengths than appear previously to have been recorded, up to 42 mm. and with as many as 7 whorls. The aperture is rounded (Fig. 1) with a very short canal, and in a well grown, non-eroded shell is only about one-third the length of the shell. The flexible, lamellar operculum fits closely into the aperture.

The spines are composed entirely of periostracum, each consisting of many parallel plates forming local extensions of the general periostracum which is formed in this way. The spines are roughly triangular, almost half as wide as they are long. Both the number of rows of spines and the length of these increases with the size of the shell, *e.g.* from 10 rows of maximum length 1.6 mm. in a shell 2.5 cm. long to 14 rows of maximum length 2.5 mm. in one 3.2 cm. long.

The shell grows by a series of obviously sudden bursts, as indicated by the conspicuous presence of areas of almost pure white shell in sharp contrast to the darker yellow or brownish older areas which are also usually overgrown with encrusting organisms (see later). A well marked flange marks the boundary between new and old areas. The relative degree of increase represented by any such burst of shell growth decreases with age and size; thus shells of lengths 1.4, 1.8, 2.7 and 3.3 cm. may recently have added approximately 1.3, 1.1, 0.5 and 0.3 whorls to the shell.

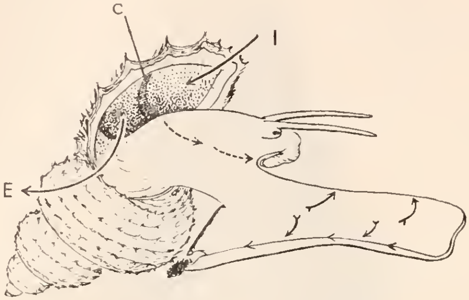

FIGURE 2. *T. cancellata,* youngest individual collected (shell length 5 mm.) before appearance of penis, with head and foot fully extended. Inhalant (I) and exhalant (E) currents, created by enlarged ctenidium (C), indicated by large arrows, route of collected food particles to mouth indicated by broken arrows, cleansing currents on surface of foot by feathered arrows, waste material accumulating at hind end below operculum.

The unusual bulk of the periostracum is indicated by the fact that the shell retains its form after complete decalcification. In three shells of between 2.7 and 3.4 cm. long so treated, the percentage by dry weight of non-calcareous matter was between 4.1 and 4.9. The great extent of the periodic bursts in shell growth may well be due to this unusually high content of periostracum, the material of which is rapidly secreted while the calcareous layers of the shell are more slowly and more continuously extended and thickened. The periodic "shoots" by which bivalves such as *Pinna* (Yonge, 1953b) increase the shell are also initially largely of protein although in this case composed almost entirely of the conchiolin matrix of the outer, prismatic layer of the shell valves.

Despite its thickness, the periostracum is soft and it is rare to find a shell of any size in which the spire is not eroded. The calcareous layers so exposed are often extensively bored into and superficially channeled by a variety of organisms including sponges and small annelids, and probably algae. Unless covered over by the many larger organisms which attach themselves to the shell (see later), the apical whorls may be completely lost. It seems doubtful whether the shell could provide adequate shelter for the animal for longer than the two years which, for other reasons, are postulated as the life span in this species.

Intact animal

When examined in sea water under a binocular microscope, the animal readily emerges from the shell, revealing the general appearance of head and foot shown in Figure 2 which represents the smallest animal collected, only 5 mm. long and immature without trace of the penis (the only such individual found). The foot is long and, at any rate until the urge to move as far as possible upwards has been satisfied, very active. It is ciliated dorsally, material being carried laterally into the grooves which run backward from either side of the opening of the pedal gland which extends across the broad anterior end of the foot. Cilia in these grooves beat posteriorly so that sediment from the foot collects in mucous masses at the posterior end of the foot beneath the operculum (see arrows in Figure 2).

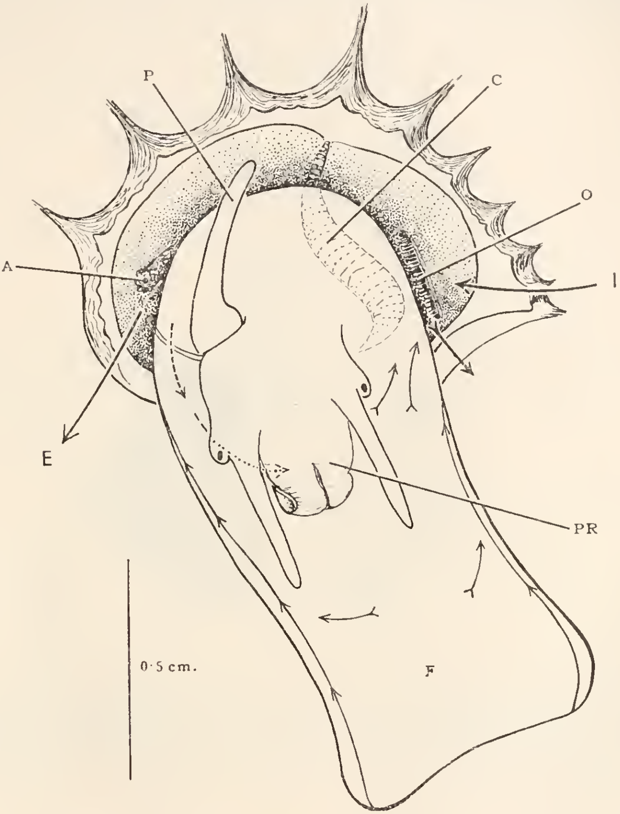

FIGURE 3 *T. cancellata*, animal extended out of shell showing foot, head and opening into mantle cavity. A, anus; C, ctenidium (viewed largely through body); F, foot; O, osphradium; P, penis; PR, proboscis. Plain arrows, respiratory and feeding current; broken arrows, food current to mouth (passing under right tentacle); feathered arrows, rejection currents.

The head bears a pair of long tentacles, each with an eye on a rounded basal protuberance. As described by Graham (1954) in *T. borealis* and precisely as in *Capulus ungaricus* (Yonge, 1938), the mouth lies at the end of a short grooved suboral proboscis. Powerful ciliary tracts coming from the floor of the mantle cavity extend round the right side of the head and carry particles to the proboscis (Figs. 2 and 3). This curls under and may move to one side or the other but certainly not invariably to the right as Graham states occurs in *T. borealis.* Without further evidence from the ctenidium, it was immediately possible to confirm Graham's statement that species of *Trichotropis,* like the allied *Capulus* (Orton, 1912; Yonge, 1938), are ciliary feeders.

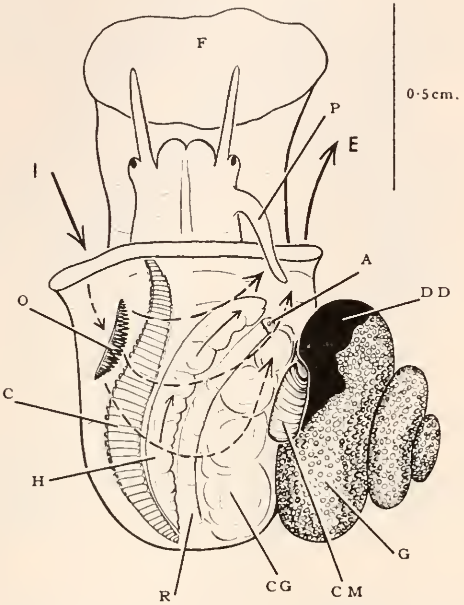

FIGURE 4. *T. cancellata,* animal removed from shell with pallial organs shown *in situ.* CG, capsule gland; CM, columellar muscle; DD, digestive diverticula; G, gonad; H, hypobranchial gland; R, rectum. Other lettering as before. Broken arrows indicate course of water currents in the mantle cavity, solid arrows, currents along surface of hypobranchial gland.

The appearance of an adult animal viewed from above as it emerges from the shell is shown in Figure 3. Here the large penis (P), *invariably present* in all but the very smallest animals, lies at the base of the right tentacle and running into it is the open seminal groove. Over this passes the food current from mantle cavity to proboscis (PR). The anterior end of the ctenidium (C) (also shown in Figure 2) appears about the middle of the roof of the mantle cavity. To the left of this (right as viewed in the figure) the dark line of the long osphradium (O) is just visible deeper within the cavity, while well to the right side the lobed margins of the anus (A) can be seen. A very powerful inhalant current (I) enters by way of the very short canal on the left side of the shell aperture; the exhalant current (E) leaves on the right, carrying with it faeces, renal products and mucus-entangled sediment from the hypobranchial gland.

FEEDING AND DIGESTION

1. Mantle cavity

In correlation doubtless with its food-collecting function, the mantle cavity is unusually deep and the contained organs, otherwise those of a typical mesogastropod, are enlarged. Their general disposition when revealed after removal of the animal from the shell, first with the mantle intact and then with this opened along the right side, is shown in Figures 4 and 5, respectively. Omitting for the time being reference to the reproductive ducts, the organs of the pallial complex—osphradium (O), ctenidium (C) and hypobranchial gland (H)—followed by the rectum (R) lie, roughly parallel to one another from left to right, along the roof. As indicated by the broken arrows in Figure 4, the water current created by the lateral cilia on the ctenidial filaments passes over each of these in turn.

Osphradium. This organ is unusually large, almost half the length of the enlarged ctenidium. It is monopectinate (not bipectinate as Graham describes it as being in *T. borealis*) and consists of some 30 filaments coming off on the right of the axis. Each is deeply pigmented on the frontal surface and cilia carry particles across its surface towards the ctenidium. The position of the osphradium is typical, *i.e.,* in the region where the heavier particles carried in the inhalant stream fall out of suspension. It also marks the point of division between the ciliary currents which carry heavier particles across the floor of the cavity and those which are retained in suspension and are carried across the roof. The significance of this is discussed below.

Ctenidium. This extends in a somewhat sinuous curve from near the margin of the mantle cavity into almost the deepest recesses where it terminates just short of the renal pore (RP) which discharges into the exhalant current. It is a typical pectinibranch ctenidium with the axis on the left and a single row of filaments, each with its lateral tracts of current-producing cilia supported beneath by a skeletal rod (Yonge, 1938, 1947). In a large animal (of shell length 3.5 cm.) the ctenidium consists of some 130 filaments. Small at the ends, these attain a length of 4 mm. in the middle of the series. Moreover, like the filaments of other ciliary feeding mesogastropods, *e.g.* species of *Capulus, Vermetus* and *Crepidula* (Yonge, 1938) or of *Struthiolaria* (Morton, 1951), they are not triangular but elongate, being about four times as long as they are broad. Thus without any great increase in the respiratory surface, the tracts of lateral cilia are elongated and so the volume of the inhalant current augmented. Particles suspended in this current when it reaches the ctenidium are largely intercepted by the frontal and abfrontal cilia which line their near and further edges. These cilia convey particles, some of them edible, to the tips of the filaments. In life these hang down towards the floor of the cavity on to which the particles are transferred by the agency of groups of terminal cilia some 30 μ long. As indicated by the arrows in Figure 5, all such particles are then carried round the right side of the head and thence to the under side of the proboscis (see broken arrow in Figure 3).

Hypobranchial gland. This lobulated structure occupies a considerable area down the middle of the roof of the cavity. On mechanical stimulation it secretes copious mucus and is covered with cilia which beat towards the opening of the cavity. Consequently the finest particles which pass between the ctenidial filaments are here entangled in mucus and carried to the right side of the cavity for extrusion in the exhalant current (see Figure 4).

Ciliary currents. Apart from the lateral cilia on the ctenidial filaments which create the respiratory current, all cilia in the mantle cavity of Mollusca were primitively concerned with removing particles of sediment, *i.e.,* with cleansing to prevent fouling of the respiratory chamber. In the Prosobranchia (Yonge, 1938) these cleansing currents are divisible into the following three groups:

A. Cilia on the margin of the inhalant region which convey the largest and heaviest particles which settle immediately to the exterior via the *inhalant* opening.
B. Cilia on the floor of the mantle cavity which carry medium-sized particles which settle deeper within the cavity across to the right where they are carried out through the exhalant opening.
C. Cilia on the frontal and abfrontal edges of the ctenidial filaments and on the surface of the hypobranchial glands, all on the roof of the cavity, in which the finest particles are collected and entangled in mucus and then extruded via the exhalant opening.

In ciliary-feeding mesogastropods these currents are modified so that to greater or less extent the collected material is passed to the mouth, *i.e.*, the currents become concerned with feeding. No new currents appear. Thus in *Vermetus novae-hollandiae* currents B and C only are diverted to carry food to the mouth but in *Crepidula fornicata* and *Capulus ungaricus* all three currents are so modified (Yonge, 1938).

In *T. cancellata* current A is present and unmodified (see feathered arrows in Figures 3 and 5). But the position with regard to B and C is unusual and interesting. Owing to the length and position (along the roof) of the ctenidial filaments these will form, as indicated in Figure 4, an effective partition between a narrower left inhalant, and a larger right exhalant, chamber. It follows that all but particles ejected by current A will reach the gills where all but the most minute will be carried down on to the floor of the cavity and so into current B which is solely concerned with food collection. But the finest particles which pass between the filaments on to the surface of the hypobranchial gland are there consolidated in mucus and extruded from the cavity in current C which is thus unmodified in function.

There is *no* anterior passage of particles along the free margin of the ctenidium, with or without an associated food groove along the floor of the cavity, as there is in the other ciliary-feeders mentioned. Nor in *Trichotropis* is the ctenidium directed to the right; indeed, there is the minimum of change apart from enlargement of the ctenidium and its movement to a mid-dorsal position together with elongation of the individual filaments. Nevertheless the particles collected in current B, *i.e.,* by the sole agency of the ctenidium, do appear to represent the only source of food. Graham (1954) does not describe the currents in the mantle cavity of *T. borealis*, noting only (p. 131) that particles from the inhalant current "fall on to and travel across the floor of the mantle cavity" and so are conveyed to the proboscis. But later he states (p. 140) that the trichotropids "may gather food with their proboscis as well as collect it out of the water current maintained through the mantle cavity." But in *T. cancellata,* of which many hundreds of specimens were observed, there is certainly no evidence that the proboscis is ever in a position to take in any material other than what is passed to it in current B from the ctenidium by way of the floor of the mantle cavity.

The position of the osphradium in relation to the three currents will be noted. Like the ctenidium, this organ is enlarged, and surely because of the increased current produced and so greater entry of sediment into the mantle cavity. The chemo-receptive powers which the osphradium comes to possess in the carnivorous neogastropods have no relevance to the needs of a ciliary-feeder and the contention (Yonge, 1947) is here reiterated that the osphradium is primarily a tactile organ concerned with estimation of the amount of sediment which enters the all-important respiratory chamber. For that reason it is enlarged in animals such as the trichotropids in which the inhalant current, and so the incoming content of sediment, is increased.

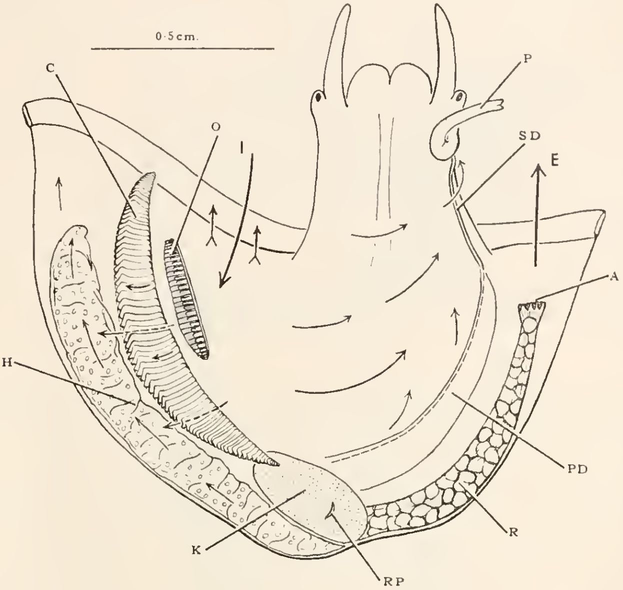

FIGURE 5. *T. cancellata,* anterior end with mantle cavity opened along right side. K, kidney; PD, pallial reproductive duct; RP, renal pore; SD, sperm duct (open groove). Other lettering as before. Rejection currents on left floor of mantle cavity (feathered arrows), feeding currents on right leading over sperm duct to proboscis under head.

2. *Alimentary canal*

Foregut. As described by Graham (1954) for *T. borealis,* cilia within the groove of the proboscis carry mucus-laden food masses up to the mouth where they are grasped by the radula and passed back through the buccal cavity. There are no jaws. A pair of bag-like salivary glands opens at the base of the cavity by short and wide ducts. Graham states that those of *T. borealis* secrete an almost pure mucus for the lubrication of the radula.

The primitive prosobranch oesophagus has been described by Graham (1939) as consisting of an anterior oesophagus, on the roof of which runs a pair of folds enclosing a ciliated dorsal food groove, a mid-oesophagus which bears lateral pouches in which digestive enzymes are secreted and a posterior and purely conducting region. He states that the glandular mid-oesophagus is absent in style-bearing prosobranchs.

In *T. cancellata* both anterior oesophagus, with its powerfully ciliated dorsal food groove, and a posterior oesophagus with ridged and ciliated walls are present. But as Professor Graham, who has compared sections of *T. cancellata* with his of *T. borealis,* agrees, oesophageal glands are much better developed in the former and have a characteristic epithelium absent in *T. borealis.* He raises the question as to whether this should be reflected in the classification, but this must be left for subsequent workers to decide.[1] What is certain is that the animal possesses both oesophageal glands *and* a crystalline style. Indeed, apart from the presence of the oesophageal glands, conditions throughout the gut generally in *T. cancellata* closely resemble those in *Capulus ungaricus* (Graham, 1939, 1954).

Stomach. When this is opened the first thing visible is the style. In an animal 3.5 cm. long, this is some 9 mm. long and about 1 mm. in diameter. A great deal of material is usually found embedded in the head which normally consists of a soft brownish mass. There may also be a thick central core of such material. While initially firm enough, the style very rapidly softens, which must explain Graham's failure to find it in *T. borealis* where he states there is a style-sac but with no style.

The appearance of the stomach when opened is shown in Figure 6. Owing to the effects of torsion, the oesophagus (OE) opens at what is now the posterior end of the stomach and the intestine (I) leaves at the anterior extremity. As noted (but not figured) by Graham (1954) for *T. borealis* and figured by him in *Capulus ungaricus,* two large ducts from the digestive diverticula (D) open one beside the entrance of the oesophagus, the other at the base of the intestinal groove (IG). The area between is finely ridged with cilia beating towards the intestinal groove. Cilia beat away from the openings of the ducts but these are mobile and food particles are probably drawn in when they dilate. In *T. borealis* the diverticula contain two types of cell, one digestive and the other probably excretory (Graham, 1954). Precisely similar cells are present in the diverticula of *T. cancellata.*

The oesophageal end of the stomach is rounded with a well developed gastric shield (GS) against which the head of the style was seen to bear when initially exposed. Ciliary tracts converge to carry material entering from the oesophagus in the head of the style where it will be rotated and mixed with its enzymes. The style-sac (SS) has the usual character, being covered with a glistening surface of dense cilia and separated from the intestinal groove by the major and minor typhlosoles. The directions of the various ciliary currents are indicated by the arrows in Figure 6. Cilia on the major typhlosole (MA) push the style towards the gastric shield, those on the general surface rotate it; on the minor typhlosole (MI) and in the intestinal groove (IG) they are concerned with moving rejected material, with much mucus, into the intestine.

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[1] See Taxonomic Addendum by Robert Robertson (p. 179).

Intestine and rectum. These regions, which run very directly from stomach to anus, are solely concerned with consolidation of the faeces. They are ciliated throughout with some muscle, especially in the rectal regions where peristalsis occurs. Graham (1954) states that mucous glands occur at both ends of the intestinal region in *T. borealis* but with other gland cells, probably secreting a protein which forms the outer covering of the faecal pellets, present in the middle regions. The oval pellets so formed in *T. cancellata* may be separate or united in chains by a common outer covering. Passing in single file through the intestine, they frequently congregate in masses in the rectum (Fig. 5). The anal opening (A) is lobed.

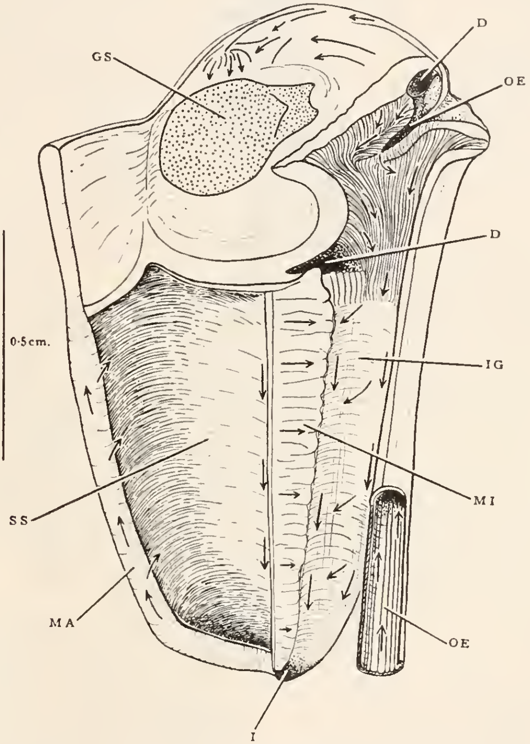

FIGURE 6. *T. cancellata,* stomach opened laterally. D, ducts into digestive diverticula; GS, gastric shield; I, opening into intestine (mid-gut); IG, intestinal groove; MA, MI, major and minor typhlosoles; OE, oesophagus; SS, style sac. Arrows indicate course of ciliary currents.

According to Graham (1939), the primitive prosobranch possessed mid-oesophageal glands which, with the apparent exception of *Adeorbis,* have been lost where a style is present, this being correlated with continuous feeding, largely on vegetable matter (Yonge, 1932). However, another exception is certainly *T. cancellata* where both glands and style are well developed, although not, according to Graham (1954), in *T. borealis.* While this difference clearly demands further examination and perhaps some change in classification, it is probably significant that *T. cancellata* (and doubtless related species) has a less specialized ciliary feeding mechanism than any other mesogastropod. The acquisition of a style may thus be relatively recent with the primitive oesophageal glands, possibly no longer secreting a protease, still retained.

Reproductive System

A most striking fact about *T. cancellata* is that all but a few very small and obviously immature individuals appear on external examination to be males. Of a series of over 600 animals, ranging in length from 1.4 to 4.1 cm., initially examined during July and August, all possessed a well developed penis. One specimen 5 mm. long (Fig. 2) had no penis and up to mid-September seven other immature specimens between 0.6 and 1.2 cm. long were collected showing various stages in the development of the penis starting with a minute papilla. But no adult examined, including very extensive samples received at Seattle in December and others at Pacific Grove in February and March, was without a large penis.

This apparent anomaly was resolved by examination of the gonad and reproductive ducts when, even on macroscopic examination, it became obvious that, apart from these few obviously immature specimens, *all* animals were in process of change from the male to the female condition or were, despite the presence of the penis, fully functional females.

The reproductive system (Fig. 7) is not otherwise unusual. The gonad (G) occupies the summit of the twisted visceral mass and from it the convoluted gonadial portion of the reproductive duct (GD) extends over the surface of the digestive diverticula to the base of the mantle cavity. By way of a short intervening renal section it is there continued as a wider and open pallial section which runs diagonally across the floor of the mantle cavity (Fig. 5, PD).

In young animals the gonadial follicles are clear green in colour and obviously testicular, but in older animals they become cream-coloured with increasingly large opaque areas denoting the formation of the large, yolky eggs. But all this time the convoluted gonoduct continues to be full of motile sperm. These macroscopic observations were later confirmed by sections.

During the early, male, phase the pallial gonoduct consists of a deep but open groove which makes connexion, by way of a wide, slit-like opening, with the seminal groove (SD) which leads to the penis. But in older animals in which the gonad is changing from testis to ovary, this region of the gonoduct hypertrophies with formation of the extensive glandular areas which, as shown in Figures 4 and 7, come to occupy the right side of the mantle roof and extend over the rectum. When fully formed this represents the capsule gland (see Fretter, 1946, for details concerning the reproductive ducts in mesogastropods). The degree of its formation is the only external indication of change of sex and even this involves removal of the animal from the shell.

These very general observations indicate that *T. cancellata* is a protandrous hermaphrodite and confirm the similar conclusions reached by Graham (1954) in his study of *T. borealis*. In a relatively small sample he had both small males and large females, the former with and the latter without a penis. Apparently the collection was made near to the time of copulation and spawning. Thus the Trichotropidae resemble the Calyptraeidae and also *Capulus* (the position in the allied but parasitic *Thyca* is obscure) in which the process of sex change has been fully described by Giesse (1915). Protandry also occurs in *Hipponix,* belonging to the related Amaltheidae (Yonge, 1960).

But in all cases hitherto described the penis is lost in the female phase. The sequence of events in species of *Crepidula* is well known while collections of *Calyptraea fastigiata* made at the same time as those of *T. cancellata* contained small males with shell diameter about 1 cm. and large females of twice that size; the former possessed the conspicuous penis found in this genus, the latter had lost all trace of this and were spawning. Even in the sessile *Hipponix* the smaller animals have a conspicuous penis lacking in the larger individuals which are all females (Yonge, 1960).

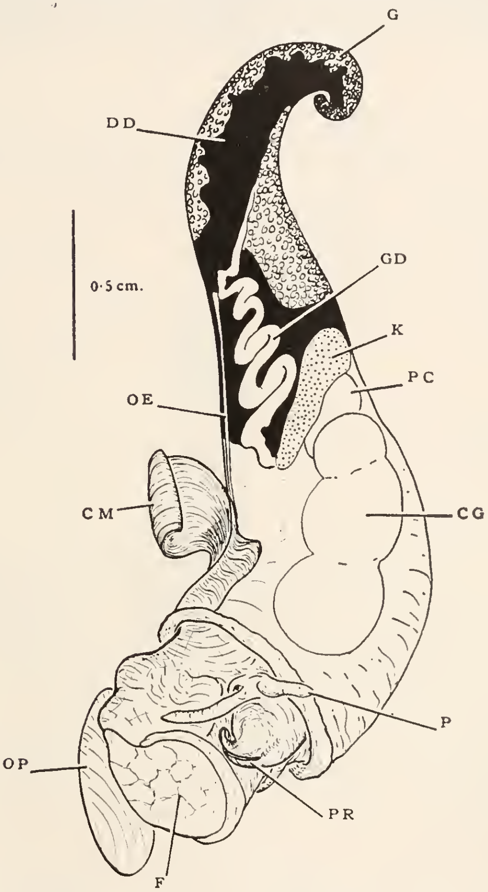

FIGURE 7. *T. cancellata,* animal removed from shell showing major organs. GD, gonadial reproductive duct; OP, operculum; PC, pericardium. Other lettering as before.

T. cancellata is the first such protandrous hermaphrodite to be described in which the penis is retained throughout life. As already noted, every individual of a large collection examined in December possessed a penis. It was then assumed that this organ would be lost before the animals became functional females in the spring. Dr. R. L. Fernald (personal communication) had already found that spawning occurs in March and April. But all members of large final samples of *T. cancellata* flown from Friday Harbor to Pacific Grove between the middle of February and the end of March, and which ranged in length from 1.5 to 4.2 cm., possessed a well developed penis. Over 100 individuals were removed from their shells and the reproductive organs examined. It was then possible, as outlined in Table I, to separate them into five groups, one of males (M) and four of females (F_1 to F_4).

TABLE I

Sexual condition of 108 specimens of T. cancellata examined between 28.ii.61 and 3.iii.61

Group	Size range in cm.	State of gonad	Gonadial duct	Capsule gland	Penis	Condition	No. of individuals
M	1.5–2.4	Empty testis (clear yellow)	Distended with sperm	No trace	Present	Male	7
F_1	2.5–4.1	Full ovary (opaque yellow)	No sperm	Developed, some capsules forming	Present	Female unfertilized	22
F_2	2.1–4.0	Full ovary	Much sperm	Capsules fully formed	Present	Females ready to spawn	43
F_3	3.5–4.2	Half-empty ovary	Full of eggs	Capsules fully formed	Present	Females spawning	6
F_4	2.8–4.2	Empty and degenerating (red)	Empty or few eggs	Usually fully discharged	Present	Females spent	30

The population thus consisted of a few ripe males (few doubtless because so much less likely to be retained in the dredge bag and to be noted when the catch was sorted) and numerous females which could be divided into four groups, (F_1) not quite ripe, (F_2) ripe and fertilized, (F_3) spawning, (F_4) spent. With one exception to be mentioned later, the females showed no evidence of recent growth and the soft periostracal spines were worn away, whereas the males had wide areas of recently formed shell with intact spines. As already noted, the state of the capsule gland is an excellent index of maturity in the female phase. After a period of steady development over the autumn and winter, it becomes an opaque white but with no sign of secreted capsules, the condition in group F_1. Gelatinous capsules then begin to be secreted (F_2); these are next liberated in spawning (F_3) and finally the gland is left yellowish and empty (F_4).

After change from the male phase, the gonad gradually becomes distended with yellow eggs (F_1); these round off prior to ovulation (F_2), following which they pack the gonadial duct where they have an average diameter of some 250 μ (F_3). The gonad is finally left flaccid and empty, apart from a few residual eggs and some reddish pigment (F_4).

The gonadial duct is distended with sperm in the active male phase and continues to contain sperm certainly well into the autumn and beginning of winter. But prior to maturity in the female phase, the duct appears empty (F_1) but then becomes filled again with sperm (F_2), presumably following copulation. After ovulation it is distended with eggs (F_3) and after completion of spawning, is empty apart from a few undischarged eggs (F_4).

From its first appearance when the shell is under 1 cm. long, the penis is present, continuing to enlarge with the growing animal. Possible reasons are the long retention of sperm within the gonadial duct or, more probably, the continuation, revealed by sections, of sperm production. In a few cases some sperm were being produced in March when the animals were ready to lay eggs. A detailed study of the gonad throughout life would be rewarding.

Although small animals, later confirmed to be males, were placed with numerous females in an aquarium tank at Pacific Grove where they appeared to live well and where some females later spawned, copulation was never observed. However, the absence of sperm in some unspawned females (F_1) and its presence in others (F_2) indicates that many females had already copulated, presumably before they were dredged at Friday Harbor. However, the position is complicated by the fact that, as reported by Mr. Jefferson Gonor, specimens left over the winter in the aquarium tanks at the Friday Harbor Laboratory also produced apparently fertile egg capsules and there seems some doubt as to whether males were present. This also requires further examination. It is possible that late sperm produced at the end of the second year may permit self-fertilization. Although Wyatt (1960) found no evidence of self-fertilization in the Calytraeidae, conditions may well be different in *Trichotropis*.

Beginning on March 7, six animals spawned on the glass sides of the tanks at Pacific Grove. They remained immobile during this process, the individual capsules being applied to the glass by the foot and arranged spirally, although all in the one plane. The greatest number of capsules produced by any animal was 26 over a period of 12 days, the final diameter of the mass being 1.7 cm. Additions (between 1 and 4) were always made at night, so that possibly light inhibits a process which may therefore be much quicker in nature.

When laid the individual capsules are round and flat, with a diameter of about 4.5 and a height of 3.5 mm. New capsules are attached peripherally, the spawn forming a rounded mass with each capsule assuming the shape of a rounded pentagon. Apart from the attached surface, which is a little thinner, the wall is about 60 μ thick. The yolk-laden and fertilized eggs, some already cleaving when first observed, are contained in a thin membrane which is possibly formed in the gonadial duct. The number of contained eggs varies very greatly, from perhaps a hundred to an occasional empty capsule. But there was little opportunity for following development which did not appear to proceed normally. This would best be observed in naturally deposited capsules.

The capsules of *T. borealis* from Greenland have been described and figured by Thorson (1935). They are rounded and between 2.75 and 4.75 mm. in basal diameter and are deposited on empty bivalve shells in clusters of only 2 to 4. Up to 13 embryos were found in each capsule but this was in mid-July with development almost completed. All capsules had an irregular exit hole through which young had already escaped. These emerged in the crawling stage and possessed peculiar conchiolin membranes running in spirals around the whorls. Thorson also describes the capsules, up to 13 in a group, and the larvae of *T. conica* which are attached to sabellid tubes.

In *T. cancellata* the spawning period certainly covers the months of March and April as observed by Dr. R. L. Fernald. It may well start in mid-February, while Mr. Gonor found a few specimens still depositing capsules in the tank at Friday Harbor as late as May 13. The three months from mid-February probably represent the full extent of the breeding season around Friday Harbor.

After spawning the great majority of animals die. In some cases the operculum has already become partially resorbed, while macroscopic examination of the tissues of the visceral mass immediately after spawning reveals widespread degeneration in both ovary and digestive diverticula. Although only a few of the animals received from Friday Harbor actually spawned, and even then development did not appear to be normal, the majority of the others only lived a few weeks or even days. Death in many cases had been preceded by ovulation, the ovary being in the same flaccid, degenerating state as in animals that had spawned. In some, death was obviously due to parasitism by small reddish trematode redia but usually it appeared to be natural. After ovulation the life span would seem normally to be completed. On May 13, Mr. Gonor reported the presence of 198 dead and only 36 survivors of the population which had been left in the tank at Friday Harbor. Some of the latter were spawning and others may possibly have been males. It is just possible that a few females which do not spawn may survive into a third year of life. One animal 3.2 cm. long, showing recent growth of shell, was encountered in March. This was a female and very dubiously ready to spawn. But it should be noted that at the end of two years the shell is usually so much eroded, especially at the apex, that survival for a further year would be impossible.

LIFE-HISTORY

From the information already recorded the course of the life-history of *T. cancellata* may be deduced, the salient facts being indicated in Figure 8. Spawning occurs between mid-February and mid-May with production of animals which grow relatively rapidly and begin to assume male characters during the late summer. This is indicated by the absence of a penis in the specimen (Fig. 2) 0.5 cm. long taken in late July and its presence in increasing size in animals up to 1.2 cm. long found in late August and mid-September. Growth continues over the winter, with accompanying enlargement of the penis and development of the testis, so that at the end of the first year of life animals, now between 1.5 and 2.4 cm. long (M in Table I), are functional males.

After copulation change to the female condition begins, the ovary slowly developing but with growth unchecked until mid-summer when animals reach almost the maximum recorded length (4.1 cm.). While change in the gonad is obvious, change in the pallial gonoduct (although doubtless beginning much earlier) does not become apparent macroscopically until the autumn when the capsule gland becomes an obvious feature in the mantle cavity. Over the second winter there is little or no evidence of further growth but both the ovary and the capsule gland become fully formed so that, fertilized by the one-year-old males, the animals can function as females at the end of the second year (*i.e.*, in the second spring as shown in Figure 8).

The long spawning period produces a great range in size. Thus the 610 animals measured in August, 1959, ranged in length from 1.4 to 4.1 cm. with a well marked unimodal peak between 2.8 and 3.3 cm. There was no evidence of any but a single year class (the immature male individuals being excluded). All of these animals had a fully developed penis and a yellow gonad. The 101 mature females reported on in Table I ranged in size from 2.5 to 4.2 cm. It is possible that some of the latest spawned individuals may not become functional females at the end of the second year and continue for a further year to spawn, if the condition of the shell permits them to survive so long, at the end of the third year. But for the great majority of individuals the duration of life appears to be two years, the animals functioning as males at the end of the first, and as females at the end of the second, year.

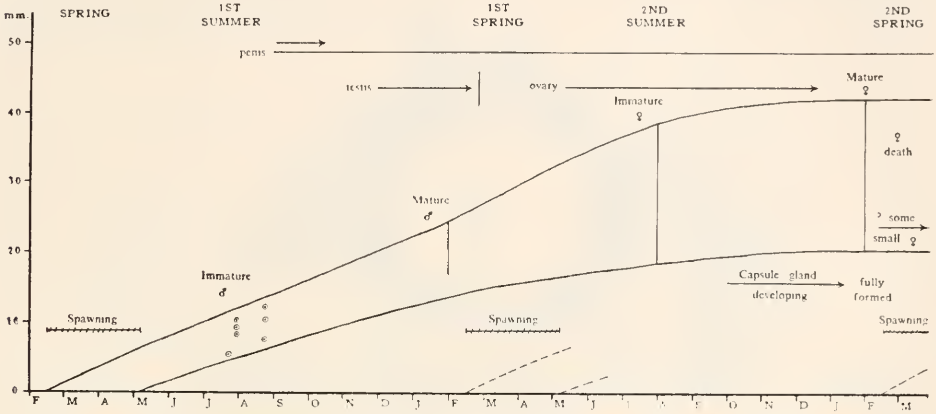

Figure 8. *T. cancellata,* probable course of life history graphically displayed. For details see text.

Habits

These can be epitomized in one sentence, *T. cancellata* always moves as high as possible and then remains quiescent. Placed on the bottom of aquarium tanks the animals crawl actively until they encounter a wall or any vertical or partly vertical surface. They then move up this to the highest available point. In the aquarium this is normally the water level where they congregate, layers deep should numbers be great. Where the surface above water level is permanently wet they will even climb several inches out of water. They remain in this position indefinitely. The few animals on the bottom which fail in their wanderings to encounter a vertical surface usually die within a few days, apparently owing to fouling of the mantle cavity with sediment from the circulating water which there accumulates.

Despite the presence of eyes, upward movement is not influenced by light, as many animals climb the dark, as the illuminated, surfaces of a tank. There is never any horizontal movement towards (or away from) a source of illumination. Movement appears to be a simple negative reaction to the pull of gravity. Having reached the highest available point—even although above water level, a state of affairs never encountered in nature by these sublittoral animals—movement ceases and is not resumed unless the animal is dislodged. Adhesion is maintained by the partly distended foot with the head and tentacles pushed as far forward as possible and the mantle cavity open. In this position, with the shell slightly raised and the mantle cavity fully open, water circulation is maintained as shown in Figure 9. The animal respires and collects suspended matter from an inhalant current largely free from sediment.

On a muddy substrate the animals immediately begin to flounder and soon become immobilized in a mass of mucus-laden mud. Even on mixed shell gravel and mud, movment is greatly hampered. *T. cancellata,* and doubtless other species of the genus, demands a firm substratum for locomotion and clear water for feeding.

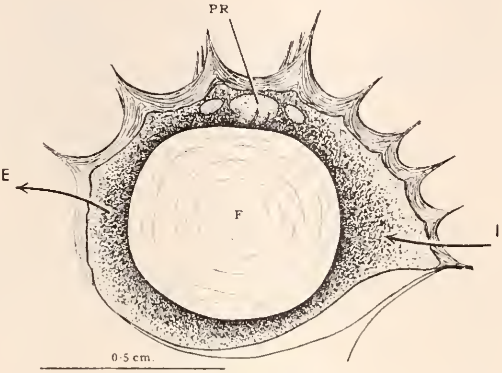

FIGURE 9. *T. cancellata,* appearance when attached to glass, quiescent and feeding. Lettering as before.

Probably no gastropod shell is so richly covered with such a variety of attaching organisms. A considerable paper could be written on the subject of this associated fauna and flora. Both the spinous covering on the shells and the habits of life encourage settlement. Within the shelter of the spines the surface is usually covered with a forest of small hydroids and encrusting polyzoans, and with chains of diatoms. A variety of small errant polychaetes and of copepods and other small crustaceans feed on the debris which collects between the spines. A small gastropod with a smooth white shell, probably a species of *Odostomia*, is not uncommon and usually in pairs.

In certain areas the shell of *T. cancellata* may be covered by a variety of large associates which in total bulk, or even singly, may have a greater volume than the shell to which they are attached (Fig. 10). These include the sponge, *Ectyodoryx parasitica* (de L.) (= *Myxilla parasitica* (Lambe)) which forms irregular yellowish masses as large as the shell; the sabellid *Eudistylia vancouverensis* (Fig. 10B) up to 4 cm. long and usually with a colony of a species of the naked two-tentacled hydroid, *Proboscidactyla*, encircling the opening of the tube; or the acorn barnacle, *Balanus balanus pugetensis* Pilsbry, which is sometimes as large as the shell and often with smaller individuals attached to a larger one. Most striking of all are the simple ascidians, notably the flat-topped *Chelyosoma productum* (Fig. 10A), of which as many as five, all larger than the shell, may be fastened to one *T. cancellata* which is completely obscured by them. Almost as common are *Styela gibbsii,* rounded and some 2.5 cm. in diameter, *Pyura haustor* (Fig. 10B) which is up to 4 cm. long, and *Boltenia villosa* with a stalked and rounded body some 2.5 cm. across. A single shell may carry representatives of all of these ascidians, together with the sabellid, so that the resultant mass may be many times the volume of the shell to which all are directly or indirectly attached (Fig. 10).

While the hairy shell certainly provides an admirable micro-habitat for many small encrusting and browsing animals, the extent of attachment by larger as well as smaller animals gives confirmatory information about the habits *in situ* of *T. cancellata*. To permit so much settlement and enable the attaching animals to grow so large, the snails must live fully exposed and move about very little. Aquarium observations are thus confirmed.

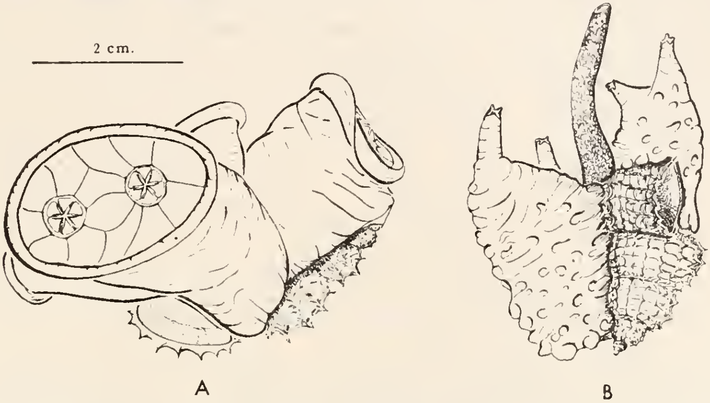

FIGURE 10. *T. cancellata* with attached organisms. A, shell largely obscured by four individuals of the simple ascidian, *Chelyosoma productum;* B, shell carrying two individuals of the ascidian, *Pyura haustor,* and between these a specimen of the sabellid worm, *Eudistylia vancouverensis.*

These snails may be envisaged as moving as high as possible on the irregular surface of the shelly bottom they inhabit, then remaining motionless with mantle cavity open for feeding and respiration. The shell with its dense coating of bristles will then present the maximum of well-protected settling surface. Movements of the foot appear sufficient to prevent the larger attaching organisms from growing over the shell aperture. Dislodgement by water movements or by the activities of larger animals will provoke renewed upward movement. Because the attaching sponges—tunicates, sabellids and barnacles—are also suspension feeders, the habits of the snail are of major advantage to them.

DISCUSSION

Two matters remain for final discussion. First there is the significance of *T. cancellata* as a biological indicator of a certain type of substrate, second the light thrown by consideration of this, and related, species on the probable course of evolution of mesogastropod limpets.

The complicated topography of the water passages between the San Juan Islands, together with the great variations in depth and in the local force of tidal currents, produce a range of bottom conditions possibly unsurpassed in any similar area. The scouring action of tidal currents in restricted passages produces the hard bottom occupied by a characteristic epifauna of largely sessile animals such as hydrocorallines and *Balanus nubilis;* maximum deposition on the bottom of deep pits or of sheltered embayments produces a soft mud substratum inhabited by suitably adapted members of the infauna. Every possible gradation between these two extremes appears to occur.

Amongst ciliary suspension feeders, many bivalves burrow in the mud while beds of *Modiolus modiolus* cover extensive areas of relatively firm bottom. In restricted areas of pure shell gravel *Glycymeris subobsoleta* is effectively the sole inhabitant, being the only bivalve which can exploit this habitat. But in certain areas where bottom currents are so powerful as to allow the unstable accumulation of little more than mounds of empty, usually bivalve, shells, an environment is produced offering unique opportunities to *T. cancellata*. No bivalve (or gastropod) can burrow here, while the substrate is too unstable for the establishment of beds of *Modiolus*. This instability also prevents successful settlement of sessile suspension feeders. But *T. cancellata* can move about freely, making inevitable progress to the highest available point where its ciliary feeding mechanism can function with maximum efficiency, the animal remaining motionless until dislodged. It then resumes upward movement. This unusual combination of mobility with a stationary habit while feeding perfectly fits this animal for life on a firm but unstable substratum. It also, as already described, renders it an ideal object of settlement by a wide variety of epifaunal suspension feeders.

The statement by Thorson (1935) that the egg capsules of *T. borealis* are laid on empty shells indicates that this species occupies a similar habitat which is probably true for all species of the genus. *T. cancellata* does extend, although in diminishing numbers, on to bottoms of mixed shell and gravel although the precise limits of its distribution can only be determined by grab samples. As an indicator of bottom conditions, this species can take its place with other sublittoral mesogastropods such as *Turritella communis,* a ciliary feeder which burrows in a bottom of stiff mud with some gravel (Graham, 1938; Yonge, 1946) and *Aporrhais pes-pelecani,* a deposit feeder which burrows in muddy gravel, and *A. serresiana* found somewhat deeper within bottoms of softer mud, the two species hardly overlapping (Yonge, 1937).

In the Mesogastropoda the limpet form and habit has been evolved in the families Capulidae (including *Capulus* and *Thyca*), Calyptraeidae (including *Calyptraea* and *Crepidula*) and Amaltheidae (including *Hipponix*). Unlike the universal habit in the far more numerous archaeogastropod limpets, none feeds by radular scraping but by ciliary currents (*Capulus, Calyptraea, Crepidula*), by the proboscis (*Hipponix*) or parasitically (*Thyca*). This is associated with a general tendency towards a sessile habit finding ultimate expression in *Hipponix antiquatus* which becomes permanently fixed early in post-larval life (Yonge, 1953*a*, 1960) and in the parasitic *Thyca*. The nature of the reproductive system, which involves internal fertilization and formation of complex egg capsules, raises problems absent in archaeogastropod limpets such as *Acmaea* or *Patella* where gametes are discharged freely into the sea. The difficulties presented to a sessile animal by the need for internal fertilization are largely met by the occurrence of protandrous hermaphroditism, with the animal still active in the early male phase (*Capulus, Calyptraea,* and some species of *Crepidula*), by the formation of chains (*C. fornicata* and other species of *Crepidula*), or by the presence of an unusually large penis (*Hipponix*).

This study of *Trichotropis* throws some light on how these mesogastropod limpets may have evolved. The pectinibranch ctenidium has been enlarged to form an organ of feeding as well as of respiration (though with modification of only one of the three cleansing currents in the mantle cavity). The animal is passive when feeding. In other words, evolution of a ciliary feeding mechanism and of a passive habit presumably preceded evolution of the limpet form which is that best fitted for sedentary existence. It provides maximum surface of attachment and minimum surface against which dislodging forces can act. Conditions in *Trichotropis* further reveal that protandry may also precede the assumption of the limpet form, although accompanying assumption of some measure of the limpet habit. It may reasonably be assumed that the mesogastropod limpets, amongst the most interestingly modified members of an order exhibiting unparalleled capacity for adaptive radiation, evolved from animals very similar in form and habits to *Trichotropis.*

Taxonomic Addendum

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The anatomical and functional differences between *Trichotropis cancellata* Hinds and *T. borealis* Broderip & Sowerby reported above by Yonge are in accord with a very recently proposed taxonomic grouping, in which one of these species is placed in a different genus. T. Habe (1961, Coloured Illustrations of the Shells of Japan (II). Osaka, Japan (publ. Hoikusha). P. 36 and appendix pp. 13–14) has renamed *Ariadna* Fischer, 1864 (*non* Audouin in Savigny, 1826) *Ariadnaria*, ranking it as a genus. The type species of *Ariadnaria* Habe and of *Ariadna* Fischer is *Trichotropis borealis,* by monotypy (Fischer). *T. cancellata* can be grouped in *Turritropis* Habe (1961, *ibid.*) because conchologically it much more closely resembles the type species of this taxon [*Trichotropis cedo-nulli* A. Adams] than it does the type of *Trichotropis*, s.s. [*T. bicarinata* (Sowerby)]. *Ariadnaria* and *Turritropis* may be ranked as genera distinct from *Trichotropis,* s.s., or grouped as subgenera within *Trichotropis,* s.l.

Summary

1. *Trichotropis cancellata* Hinds is a member of the Trichotropidae which, with the Capulidae and Calyptraeidae, constitutes the Superfamily Calyptraeacea. As in other species of the genus, the shell is covered with unusually thick periostracum prolonged into characteristic spiral rows of spines. Older shells are always deeply eroded apically.

2. The mantle cavity possesses the typical organs of a mesogastropod. Particles collected by the enlarged, but not otherwise specialized, ctenidium are carried by ciliary currents, representing modification of only one out of three groups of

cleansing tracts, under the right side of the head to the grooved proboscis. Enlargement of the monopectinate osphradium is to be associated with greater intake of sediment in the augmented inhalant current.

3. The gut is unusual in possessing both a glandular region in the oesophagus and a crystalline style. The former, primitive, structure is usually lost when a style is present. This provides further evidence of the relatively recent adoption of the ciliary feeding habit.

4. Like other Calyptraeacea, *T. cancellata* is protandric. The penis first appears in animals over 0.5 cm. long. Spawning (at Friday Harbor) is probably from mid-February to mid-May, the animals functioning as males when one year old and between 1.5 and 2.4 cm. long.

5. During the second year, change to the female condition occurs with modification of the pallial reproductive duct to form a capsule gland, the eggs now produced by the gonad being deposited in gelatinous capsules when the animals are two years old and reach a maximum length of 4.2 cm. The great majority, if not all, then die.

6. The process of egg laying, although not of copulation, has been observed.

7. Unlike allied animals, including *T. borealis*, the penis is retained and enlarges throughout life. This may be due to long retention of sperm in the gonadial duct, more probably to continuation, sometimes until egg-laying, of some production of sperm in the gonad. There is evidence that self-fertilization may occur.

8. Habits involve the simple process of moving as high as possible and then remaining quiescent while feeding on suspended matter drawn in with the increased inhalant current. Only when dislodged is activity resumed. Despite the presence of eyes, habits appear uninfluenced by light.

9. *T. cancellata* is thus admirably adapted for life on a firm but unstable substrate of dead, largely bivalve, shells. It flounders on soft substrates. Probably no gastropod shell is so richly covered with such a diversity of attaching organisms. Both the spinous covering and the habits encourage settlement of organisms up to the size of tube worms, barnacles and ascidians, the total bulk of which may greatly exceed that of the shell.

10. *T. cancellata* is an indicator of a restricted type of bottom condition. Much may be learnt from it about the manner in which the limpet form and habit has been acquired in the Mesogastropoda. Assumption of the habit of life, represented here by ciliary feeding and protandry, clearly precedes that of the limpet form.

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ON THE REPRODUCTIVE CYCLE AND BREEDING HABITS OF TWO WESTERN SPECIES OF HALIOTIS [1, 2]

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The genus *Haliotis* (Linnaeus) is composed of some 75 species distributed throughout the world. Members of this genus occur from subarctic to antarctic waters, but they are most abundant in temperate and tropical waters as common inhabitants of rocky intertidal and subtidal zones. Because these animals have been of commercial value since ancient times much has been written about their natural history, beginning with Aristotle (see Crofts, 1929). Chance observation of spawning and sporadic examination of the condition of gonads have been recorded by many investigators and fisheries biologists. However, no systematic investigation has been made of the reproductive cycle and breeding habits of haliotids, except for the recent study of gametogenesis in *H. lamellosa* from the Mediterranean Sea (Bolognari, 1954).

In the present study, changes in the size of *Haliotis cracherodii* and *H. rufescens* gonads relative to body size were followed throughout the year.

MATERIALS AND METHODS

H. cracherodii and *H. rufescens* were collected at Hopkins Marine Station, Pacific Grove, California. In this area *H. cracherodii* is found attached to surfaces of overhanging rocks and crevices in the intertidal zone 3 (Ricketts and Calvin, 1948). *H. rufescens* commonly occurs attached to rocky surfaces, from the lowest tide mark down to a depth of approximately 30 feet. Specimens were therefore collected by diving with the aid of self-contained underwater breathing apparatus (SCUBA).

Ten animals of each species were collected monthly and brought to the labora-

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[2] We are indebted to Dr. Mitsuhiro Nozaki, Department of Soil and Plant Nutrition, University of California, Berkeley, for translation of K. Tago's (1931) article, to Professor L. R. Blinks, Director, Hopkins Marine Station for providing facilities for this work, and to Mr. D. Leighton for permission to quote unpublished data.

[3] As of September 1961, at the University of Shiraz, Shiraz, Iran.

tory for analysis. Measurements of the shell and soft body parts characterizing the population investigated are summarized in Table I. Of 130 *H. cracherodii* collected, 54 were females and 76 males, and of 140 *H. rufescens,* 79 were females and 61 males. These samples, however, are too small to establish a significant difference in the frequency of the sexes.

The gonad—yellow cream-colored in males, and green in females—occurs as a sheath covering a curved, horn-shaped structure, generally referred to as the conical appendage. The center of the cone is occupied by the brown-colored hepatic tissue. This appendage runs along the right side of the shell muscle. In order to expose the conical appendage, the shell was separated from the rest of the animal by a large spatula. The conical appendage, severed near the stomach and immediately frozen in a Deep Freeze at −25° C., was cross-sectioned with a sharp knife at 2.5-cm. intervals from the apex (Fig. 1).

The perimeters of the gonad and hepatic tissues were traced directly on transparent thin plastic of uniform weight. These tracings were cut from the sheet and weighed. The areas of the tissues at the specific cross-sections were finally determined from a standard curve which related the area of the mentioned plastic to its weight.

The size of the gonad and hepatic tissues varies with variations in size of the animals. In order to observe seasonal changes in the quantity of these tissues, the variation related to size *per se* must be taken into account. The index chosen for this purpose is the ratio of the area of the gonad (or hepatic tissue), at any given cross-section of the cone, to the shell length, multiplied by a hundred.

H. cracherodii

The monthly mean values of gonad and hepatic indices, determined from the cross-section of the conical appendage 5 cm. from the apex, are plotted in Figures 2 and 3, respectively. The 95% confidence limits are also plotted for each point. In Figure 4, the hepatic index is superimposed on the gonad index.

These figures show that *H. cracherodii* exhibits an annual reproductive cycle. The gonads enter a period of growth beginning in March, attaining maximum size towards the end of May. Spawning begins in June and continues to October for most members of the population. From November to March the gonads enter a quiescent period and a minimum size is reached. The size of the hepatic gland

TABLE I

Measurements of shell and soft body parts of H. cracherodii and H. rufescens used in the determinations reported

Measurement	*H. cracherodii*		*H. rufescens*	
	Mean	Range	Mean	Range
Total wet weight in g.	587.2	240.0–1020.0	1410.6	622.0–2330.0
Soft body weight in g.	334.6	120.0–581.1	844.7	380.0–1370.0
Shell weight in g.	252.4	80.7–428.0	565.9	194.1–864.5
Shell length in cm.	14.0	10.8–16.5	19.0	15.0–22.5

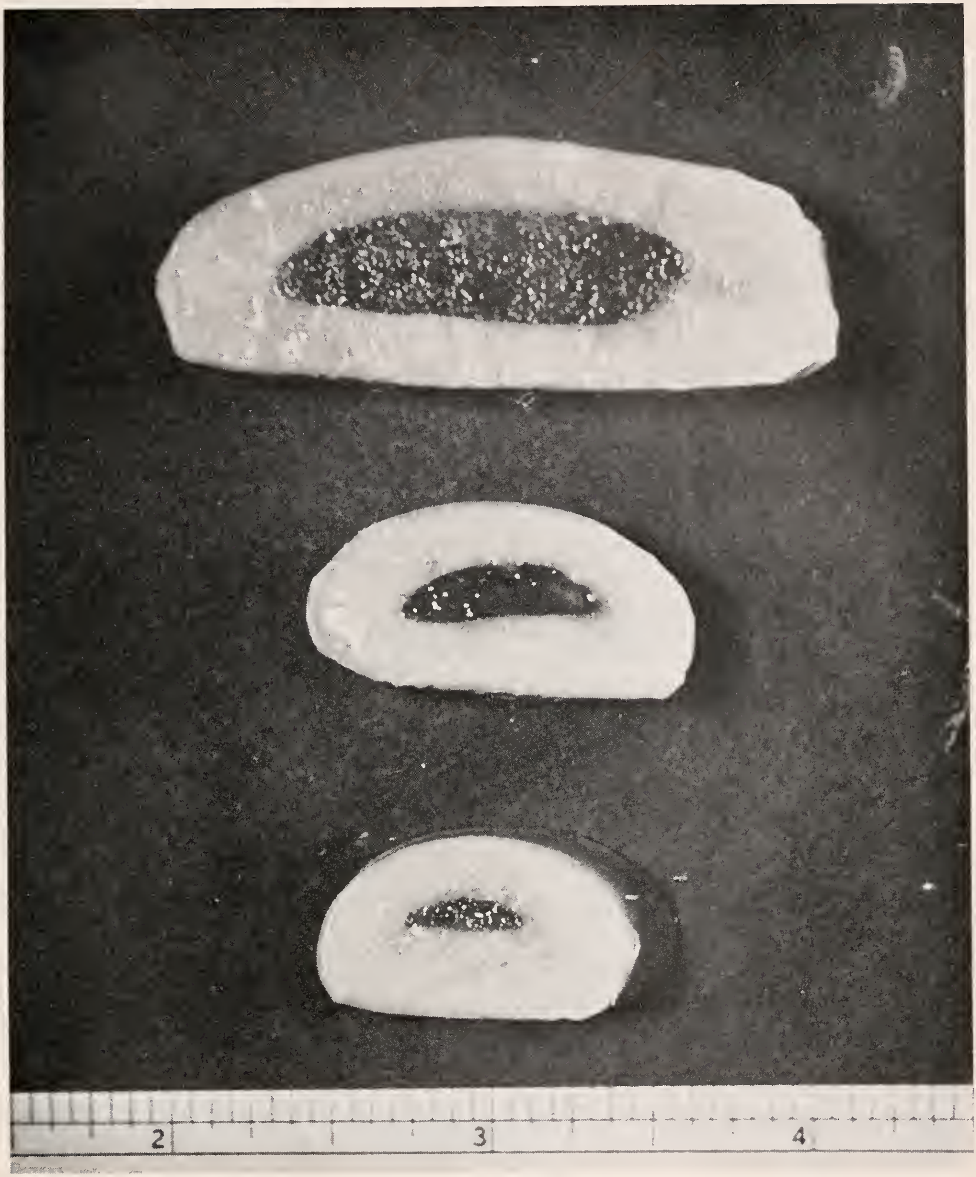


FIGURE 1. Cross-sections of the conical appendage of *H. rufescens.* The dark central region is the hepatic tissue and the light periphery is the gonad tissue. Scale in mm.

exhibits a distinct inverse relation to gonad activity. It undergoes a period of growth beginning in August and reaches its maximum by February. This period corresponds to gonadal quiescence. From February to April there is a precipitous drop in the size of the hepatic gland and this corresponds to the growth period of the gonad. Finally the hepatic index attains a minimum value between April and

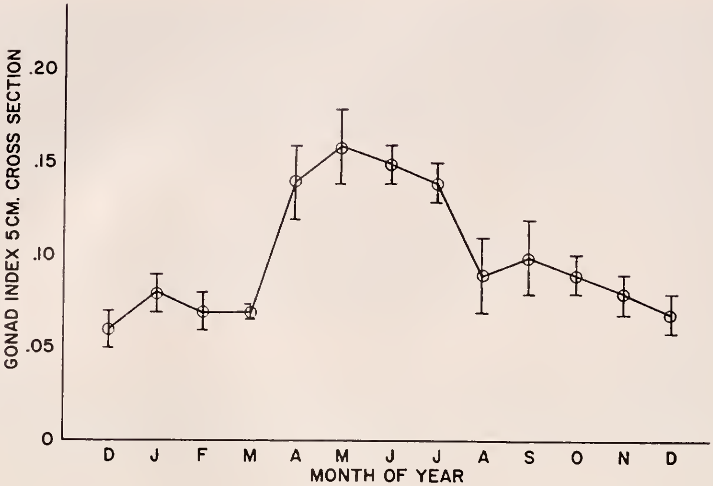


FIGURE 2. Gonad index of the abalone, *H. cracherodii,* for the period December, 1956 to December, 1957. The circles are the mean values and the vertical lines are the 95% confidence limits.

August, during which the gonads are at the height of their activity. These curves suggest that materials are mobilized from the hepatic gland for the growth of the gonad.

Examination of gametes throughout the year showed active sperm and ripe eggs from June to October. Ripe gametes obtained by gonadal biopsies, however, could not be fertilized.

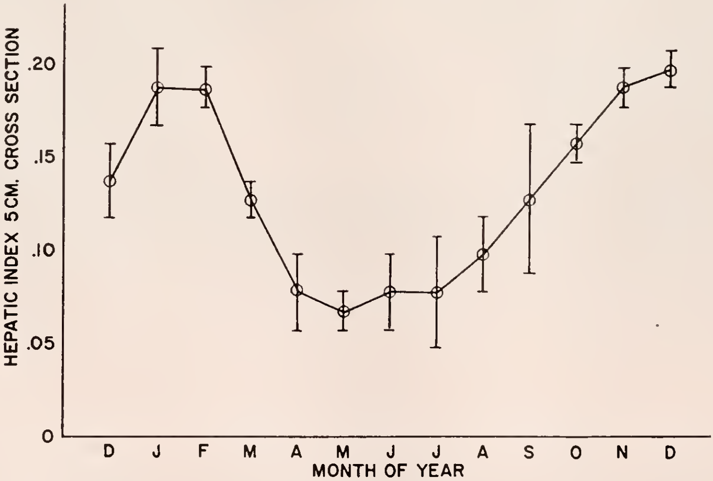


FIGURE 3. Hepatic index of the abalone, *H. cracherodii* for the period December, 1956 to December, 1957. The circles are the mean values and the vertical lines are the 95% confidence limits.

It is of interest to point out that similar results were recently obtained for *H. cracherodii* at Point Dume, in Southern California (Leighton and Boolootian, to be published). This is not only a confirmation of the above observation but also indicates that the cycle observed at Pacific Grove is not restricted to this latitude.

H. rufescens

The monthly mean gonad and hepatic indices of *H. rufescens* are presented in Figures 5 and 6. These figures also show the 95% confidence limits of the mean. Figure 7 presents the hepatic and gonad indices together.

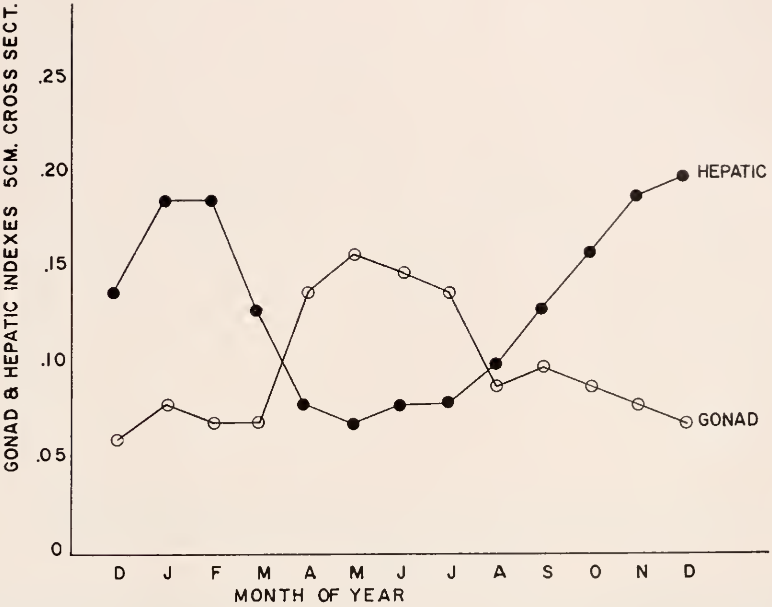


FIGURE 4. The annual gonad and hepatic cycles for *H. cracherodii*. Note inverse relationship between the gonad and hepatic cycles.

In contrast to *H. cracherodii* this organism does not exhibit a distinct reproductive cycle; high gonad and hepatic indices are maintained throughout the year. Monthly examination of the gonad tissues showed ripe gametes at all times, although it was not possible to fertilize the eggs. The inverse relation between gonad size and hepatic indices also holds for this species.

On the basis of the data at hand it is not possible to state whether the fluctuations observed in gonad and hepatic indices represent true seasonal fluctuations. The above data indicate that *H. rufescens*, when considered as a population, breeds throughout the year. This view is further strengthened by evidence presented in Table II, which shows that recently metamorphosed individuals have been recovered from kelp holdfasts throughout most months of the year (Leighton, personal communication).

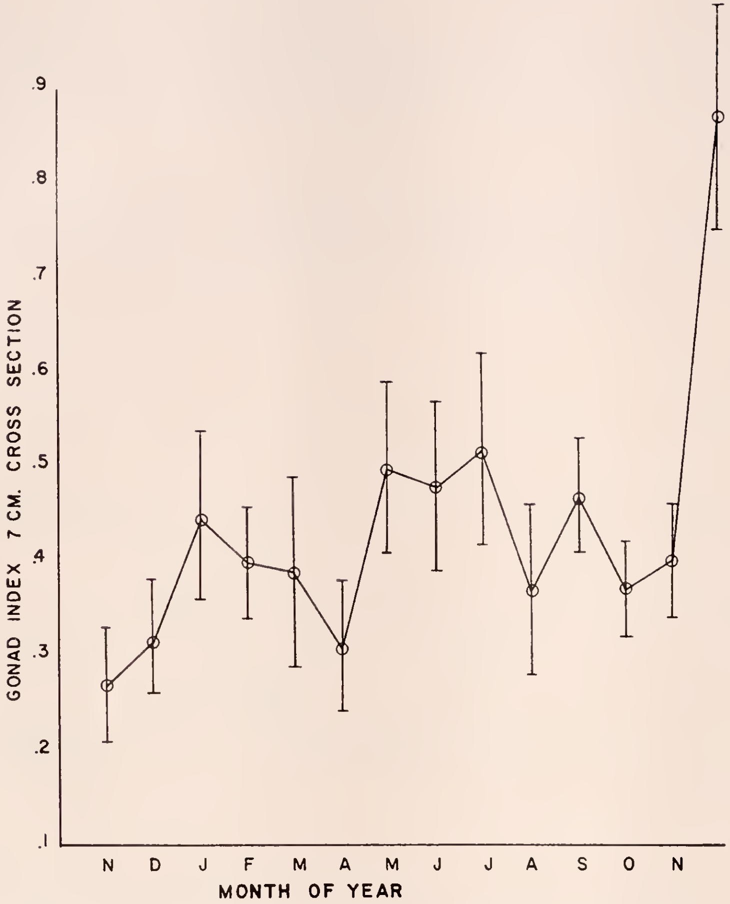


FIGURE 5. Gonad index of the abalone *H. rufescens* for the period November, 1956 to November, 1957. The circles are the mean values and the vertical lines are the 95% confidence limits.

DISCUSSION

Table III is a graphic summary of the breeding seasons reported in the literature for eight species of *Haliotis*, from various parts of the world. Various lengths of breeding season have been reported for *H. rufescens* (Bonnot, 1930, 1940, 1948; Scofield, 1930; Croker, 1931) probably because systematic observations were not made throughout the year. As information was accumulated, the spawning period was extended, and Bonnot (1948) reported it to be from March to September.

Monthly examination of the gonads of this organism at Pacific Grove, California, disclosed mature gametes throughout the year. Furthermore, recently metamorphosed individuals have been found at all seasons in Southern California. It thus appears likely that in both localities *H. rufescens*, considered as a population, breeds throughout the year.

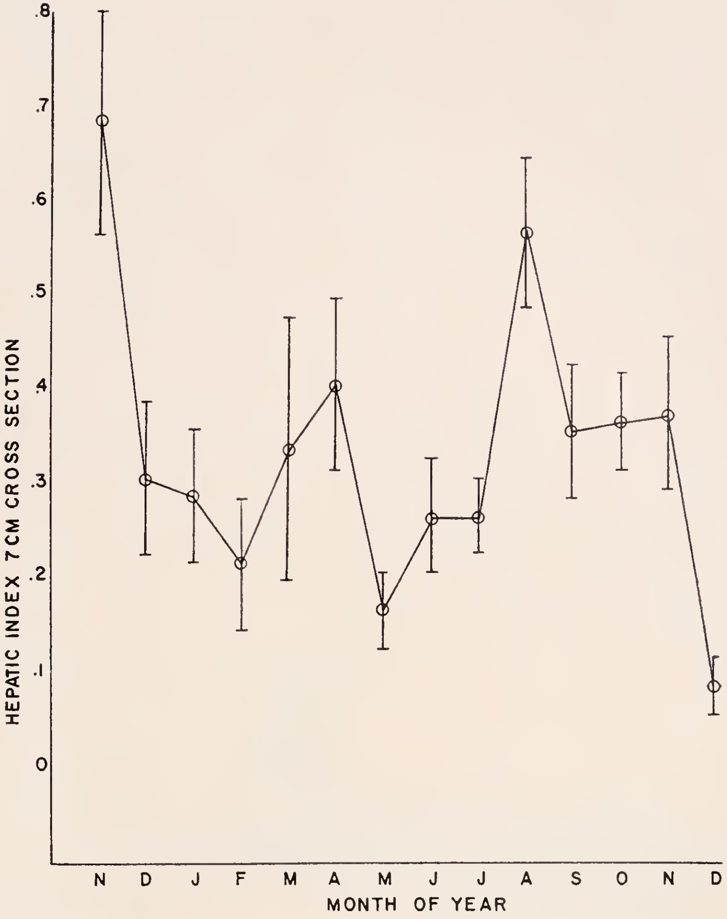


Figure 6. Hepatic index of the abalone, *H. rufescens* for the period November, 1956 to November, 1957. The circles are the mean values and the vertical lines are the 95% confidence limits.

The other haliotids seem to have a restricted breeding season. Systematic observation of the reproductive condition of *H. lamellosa* (Bolognari, 1954) at Messina in the Mediterranean Sea indicates that maturation of gametes takes place during March and April. By June the gonads of most individuals attain full maturity and spawning begins. Spawning terminates in October, at which time the gonads enter a quiescent period. *H. cracherodii* at Pacific Grove, California, similarly has a gametogenic period during March and April, the gonads being fully ripe by May and June in most members of a sample. Spawning begins in June and the gonads enter a quiescent period by October. It is interesting to point out that although *H. lamellosa* and *H. cracherodii* occur in widely separated areas, they occur at approximately the same latitude.

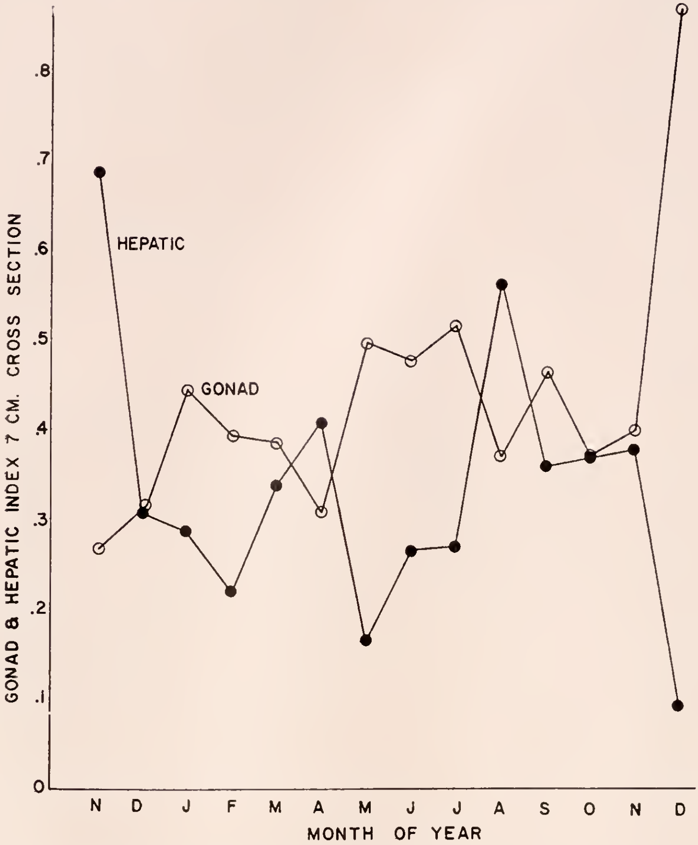


Figure 7. The annual gonad and hepatic cycles for *H. rufescens*. Note inverse relationship between the gonad and hepatic cycles, similar to that found for *H. cracherodii*.

Spawning of *H. tuberculata* in the English Channel (Wegmann, 1884; Crofts, 1929, 1937) occurs between May and September much as in *H. cracherodii* and *H. lamellosa*.

Of the four Japanese haliotids presented in Table III, *H. kamtschatkana* is a cold-water species found in Hokkaido. This organism has been observed to spawn from July through November. The other species, *H. gigantea*, *H. sieboldii*, and *H. discus*, are warm-water species occurring further south and are known to spawn later in the season (from October to December). In this group *H. kamtschatkana* has a spawning pattern similar to that of *H. cracherodii*.

The only report of breeding habits for the haliotids of the southern hemisphere is that of Graham (1941) for *H. iris* from New Zealand. He observed a single specimen to spawn in a laboratory aquarium in mid-December. Since December marks the beginning of the summer in the southern hemisphere, this period is seasonally comparable to the spawning period of *H. cracherodii*.

To summarize, with the exception of *H. rufescens*, which apparently spawns

TABLE II

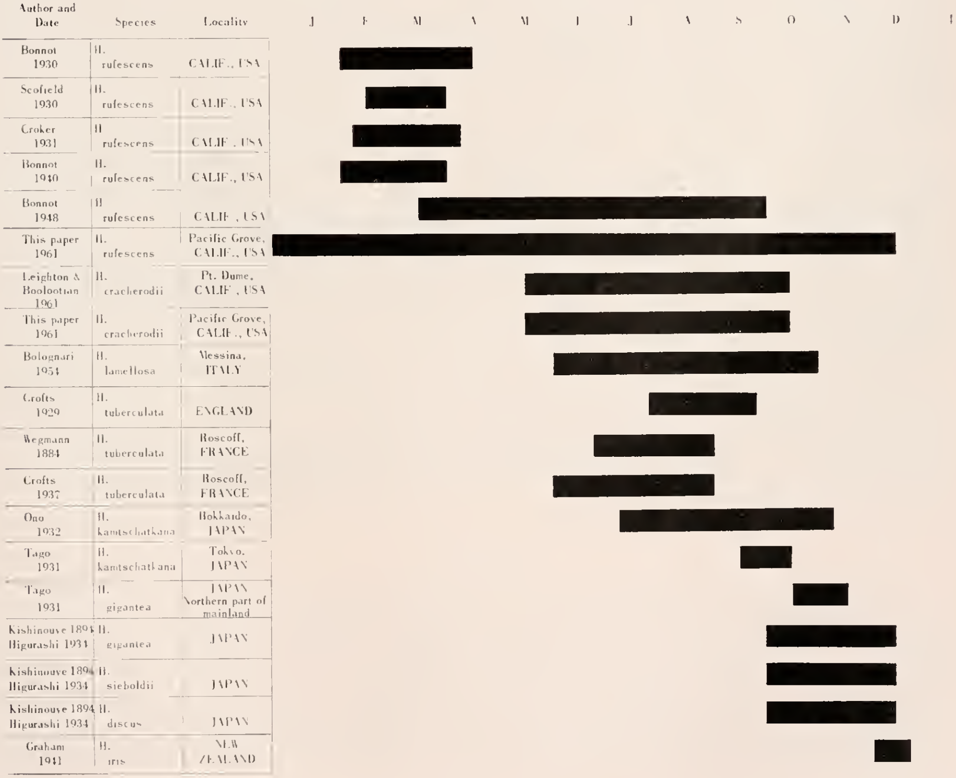
*Recovery of H. rufescens from kelp holdfasts, Macrocystis pyrifera**

Date	Size Range in mm.	Number
9-22-59	2.8–10.0	4
10-24-59	5.0–18.0	4
2-13-60	2.8–8.0	12
4-12-60	3.0–9.3	13
7-18-60	3.0–5.0	3
9-25-60	2.0–25.0	23
10-5-60	1.8–19.0	26
10-18-60	3.0–23.0	16
11-28-60	3.0–8.5	10
12-19-60	3.2–19.0	53
2-6-61	1.0–15.5	33

* All samples were collected from near Scripps Institution of Oceanography, La Jolla, California. Most specimens were found among the sporophytic fronds (Leighton, personal communication).

throughout the year, all the species of *Haliotis* listed above have a spawning period ranging from late spring to early autumn with slight modifications due to local conditions. These may therefore be considered summer breeders.

TABLE III Summary of breeding season of *Haliotis*



Author and Date	Species	Locality
Bonnot 1930	H. rufescens	CALIF., USA
Scofield 1930	H. rufescens	CALIF., USA
Croker 1931	H. rufescens	CALIF., USA
Bonnot 1940	H. rufescens	CALIF., USA
Bonnot 1948	H. rufescens	CALIF., USA
This paper 1961	H. rufescens	Pacific Grove, CALIF., USA
Leighton & Boolootian 1961	H. cracherodii	Pt. Dume, CALIF., USA
This paper 1961	H. cracherodii	Pacific Grove, CALIF., USA
Bolognari 1954	H. lamellosa	Messina, ITALY
Crofts 1929	H. tuberculata	ENGLAND
Wegmann 1884	H. tuberculata	Roscoff, FRANCE
Crofts 1937	H. tuberculata	Roscoff, FRANCE
Ono 1932	H. kamtschatkana	Hokkaido, JAPAN
Tago 1931	H. kamtschatkana	Tokyo, JAPAN
Tago 1931	H. gigantea	JAPAN Northern part of mainland
Kishinouye 1894 Higurashi 1934	H. gigantea	JAPAN
Kishinouye 1894 Higurashi 1934	H. sieboldii	JAPAN
Kishinouye 1894 Higurashi 1934	H. discus	JAPAN
Graham 1941	H. iris	NEW ZEALAND

The breeding habits of molluscs in general fall into three large categories, namely: (1) year-around breeders, (2) winter breeders which spawn between the end of autumn and the beginning of spring, and (3) summer breeders which spawn between the end of spring and the beginning of autumn. Costello *et al.* (1957) list the breeding season of 24 species of Atlantic molluscs of which 23 breed during the summer and one breeds throughout the year. For the Pacific Coast, MacGinitie and MacGinitie (1949) list 18 species of molluscs; 9 are summer breeders, 6 are winter breeders and three breed throughout the year. Of the 8 species of *Haliotis* considered in this paper, 7 are summer breeders and one breeds throughout the year. Finally, Graham (1941) lists 22 species of molluscs from the New Zealand region, all of which are summer breeders. It thus appears that 85% of the molluscs considered are summer breeders, irrespective of their geographic distribution.

It is beyond the scope of this paper to consider the variety of exogenous and endogenous factors which may affect production and release of gametes in various organisms (see Giese, 1959). It is not possible at this time to do more than speculate about the factors controlling spawning in the abalone. It may seem surprising that such closely related species as *H. rufescens* and *H. cracherodii*, occurring in the same geographic area, exhibit different breeding habits, the former breeding throughout the year, the latter for only a short season. It is possible that seasonal changes in the availability of food (general or specific) determine the period of gamete production. The intertidal species, *H. cracherodii*, is subjected to great variations in the quantity and quality of algae serving as its food, while *H. rufescens*, being subtidal, is subjected to less seasonal variation of this nature (Leighton and Boolootian, to be published).

A distinct increase in the size of the hepatic gland was observed to precede breeding in *H. cracherodii*. Subsequently, as the gonad increased in size the hepatic gland subsided. This reciprocal relation between the hepatic gland and gonad is similar to that found in *Pisaster ochraceus* (Farmanfarmaian *et al.*, 1958). It appears that the hepatic gland stockpiles nutrients essential to gamete development. Stockpiling may occur at a season when the food supply (quantitatively, qualitatively, or both) is most effective. Decisive data for or against this view are lacking.

In *H. rufescens*, no such cycle has been observed, both hepatic gland and gonad being well developed almost throughout the year. It would thus appear that nutrients required for both hepatic and gonadal growth are always available. Only observations on animals under controlled feeding conditions will enable one to determine whether food or some other factors control breeding in these species.

SUMMARY

1. The annual reproductive cycles were determined for two species of abalone: *Haliotis cracherodii* and *H. rufescens*, all found in Monterey Bay. The method consisted of tracing on transparent thin plastic the perimeter of the gonad and hepatic tissues. The tracings were cut and weighed. The areas of these tissues were determined from a standard curve which related the area of the plastic to its weight. The ratio of the area of the gonad (or hepatic tissue) to the shell length

× 100 yields an index for measuring seasonal changes in the quantity of these tissues.

2. *H. cracherodii* shows a marked summer breeding season, the rapid and precipitous spawn-out being indicated by the decline in the size of the gonad.

3. *H. rufescens* shows an ill-defined breeding season and, from reports, seems to spawn at all times of the year. The gonad size was found to vary little during the year except for an increase in winter.

4. In both species the hepatic index is inversely correlated with the gonad index.

5. A summary of the breeding seasons reported in the literature for eight species of *Haliotis* from various parts of the world is discussed.

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INHIBITION OF FERTILIZIN AGGLUTINATION AND FERTILIZATION IN ARBACIA BY FUCUS EXTRACTS[1]

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A number of substances which impair or inhibit the normal fertilization response of echinoderm gametes have been investigated. These include natural substances from the gametes themselves (reviewed in Metz, 1957), fluids from the parent animals (Metz, 1960), and various extraneous agents (Wicklund, 1954a). Of these various agents, those which inhibit fertilization without "injuring" the unfertilized egg or spermatozoa are of most interest (Wicklund, 1954a). Harding (1951) reported that crude "fucoidin," a polysaccharide from the brown alga *Fucus vesiculosus*, was such an inhibitor. That observation led to a more detailed investigation of the fertilization inhibitor from *Fucus,* designated FeInh(Fu) by the Swedish investigators (Wicklund, 1954c; Esping, 1957a, 1957b; Runnström *et al.,* 1959) and Branham and Metz (1959, 1960).

Wicklund (1954a) demonstrated that sea urchin spermatozoa were not paralyzed by FeInh(Fu) solutions and were capable of fertilizing eggs after an exposure to the extracts of up to eight hours. The same investigator also found the inhibitory effects on eggs to be reversible when the material was washed from the eggs. It was concluded that the FeInh(Fu) had to be present at the time of insemination in order to inhibit fertilization. Branham and Metz (1959), however, reported that *Arbacia* eggs were irreversibly inhibited after exposure to *Fucus* extract.

Several physical effects of FeInh(Fu) on eggs have been demonstrated by Wicklund (1954b) and Runnström and Hagström (1955). The former investigator demonstrated that exposure to the inhibitor resulted in a stiffening of the egg cortex. The latter investigators found that FeInh(Fu) prevented the swelling of the egg jelly layer which normally occurred when eggs were placed in sea water.

Lundblad (1954) found that two proteolytic enzymes extracted from echinoderm eggs were inhibited by FeInh(Fu). Esping (1957b) further investigated the effect of *Fucus* extracts on enzyme systems *in vitro* and reported inhibition of hyaluronidase, DNAase, alpha amylase and urease. She concluded that the inhibitor acted on a non-specific protein part of the enzyme.

The chemical nature of the fertilization inhibitor from *Fucus* is little known. Wicklund (1954a) recognized two kinds of inhibitors, one ("polysaccharide")

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which became inactive when oxidized by periodate, the other ("phenolic substance") which was not affected by this oxidizing agent. It was demonstrated, however, that the purified fucoidin preparations had no inhibitory effects (*cf.* Wicklund, 1954a). Esping (1957b) investigated the chemical nature of the hyaluronidase inhibitor for *Fucus* and concluded that it was probably a polyphenolic compound.

The present report is concerned primarily with further investigations of the effects of *Fucus* extracts on echinoderm spermatozoa. In contrast to the observations of Runnström and Hagström (1955), the *Fucus* extracts prepared in this laboratory prevented fertilizin agglutination of the sperm. The results presented here indicate that the sperm antifertilizin is inactivated by the agent(s) which is absorbed by the sperm. A second effect of the *Fucus* extract is to increase the rate of O_2 consumption of the spermatozoa. Despite these effects the sperm were capable of fertilizing eggs after exposure to the inhibitor. Eggs, however, fail to raise fertilization membranes or cleave when inseminated after exposure to the *Fucus* extract solutions.

MATERIALS

Extracts of the alga *Fucus vesiculosus* were prepared by a modification of the method reported by Esping (1957a, method C). Fresh *Fucus,* obtained from the intertidal zone at Woods Hole, Mass., were repeatedly extracted by soaking the moist alga in 5 to 10 changes of tap water (Fig. 1). The extract thus obtained was concentrated under vacuum at temperatures below 80° C. Air was bubbled

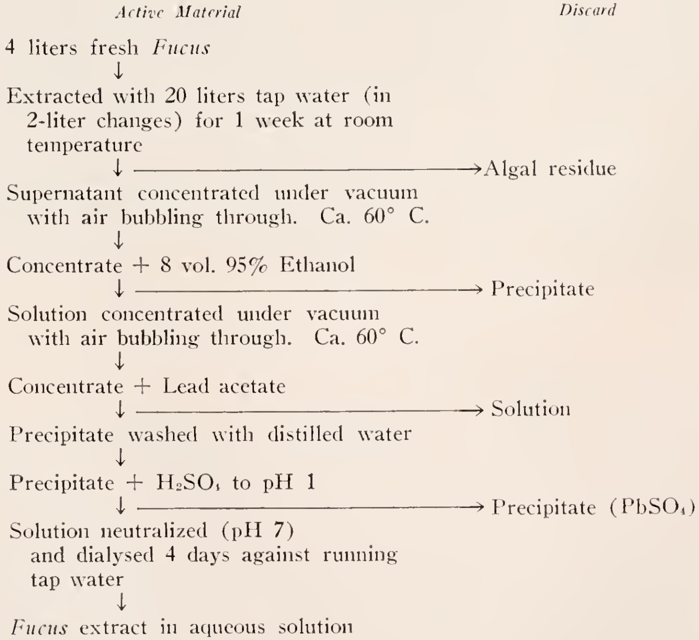
Active Material
Discard
4 liters fresh *Fucus*
Extracted with 20 liters tap water (in 2-liter changes) for 1 week at room temperature
Algal residue
Supernatant concentrated under vacuum with air bubbling through. Ca. 60° C.
Concentrate + 8 vol. 95% Ethanol
Precipitate
Solution concentrated under vacuum with air bubbling through. Ca. 60° C.
Concentrate + Lead acetate
Solution
Precipitate washed with distilled water
Precipitate + H_2SO_4 to pH 1
Precipitate ($PbSO_4$)
Solution neutralized (pH 7) and dialysed 4 days against running tap water
Fucus extract in aqueous solution

FIGURE 1. A method for the preparation of *Fucus* extract.

through the extracts to speed evaporation. The concentrated syrup was added to 8–10 volumes of 95% ethanol and the supernatant obtained after removal of the precipitate was again concentrated under vacuum. Saturated lead acetate was added to the ethanol-soluble fraction until precipitation was complete. The precipitate was collected and washed, then reacted with sulfuric acid. The precipitate (primarily lead sulfate) was removed and the supernatant neutralized with sodium hydroxide and dialysed against running sea water or tap water. In the latter instance isotonicity was obtained by mixing with an equal volume of sea water concentrated to half the original volume or diluting with more than 100 volumes of sea water just before use.

No precautions were taken to avoid bacterial contamination in the long extraction. However, upon repetition, the procedure gave consistent results. Furthermore, other methods, including extraction by boiling, gave preparations with similar properties. Therefore, it is likely that the active material is not a product of bacterial action.

The amount of dry matter in the extract solutions was determined by dialysing aliquots against distilled water and drying to constant weight at 85° C. It was difficult to redissolve the dry material and most of the activity of the preparation was not recoverable after drying. For this reason the *Fucus* extracts were kept in solution and the amount of dry matter used in the tests was calculated from the volume used.

All experiments except those dealing with specificity employed gametes of *Arbacia punctulata*. The animals were from the Woods Hole, Mass., area or from the Gulf of Mexico in the vicinity of the Florida State University Marine Laboratory at Alligator Point, Florida. Gametes were shed by the usual methods of electrical stimulation or the application of isotonic KCl to the gonads (Costello *et al.*, 1957). Sperm concentration was estimated by measuring the optical density of the sperm suspension with a Klett-Summerson colorimeter at 420 mμ. The standard curve was prepared by measuring the optical density of sperm suspensions and counting the spermatozoa in a Levy counting chamber (haemocytometer). Fertilizin was prepared by decanting the supernatant "egg water" from standing egg suspension, or by acid sea water (pH 5) extraction of the egg jelly material followed by neutralization with sodium hydroxide.

Experiments and Results

Inhibition of fertilizin agglutination of sperm

Runnström and Hagström (1955) reported that FeInh(Fu) did not agglutinate *Paracentrotus lividus* sperm or prevent sperm agglutination with homologous fertilizin. The *Fucus* extracts prepared in this laboratory were similarly tested with *Arbacia* sperm and fertilizin. One drop of dilute sperm (about 1% semen in sea water) was added to two drops of *Fucus* extract in two-fold dilution series. One drop of fertilizin was then added to the sperm mixture, and the agglutination observed macroscopically. Sperm in higher concentrations of the inhibitor remained motile but failed to agglutinate when fertilizin was added. In lower concentrations of the extract they agglutinated normally. The amount of extract necessary to inhibit fertilizin agglutination varied with different extracts. The most active extract prepared inhibited agglutination at a concentration of 7.34×10^{-6} gm. of dry material/ml. when tested with 0.5% semen. Similar inhibition of fertilizin agglutination was observed with sperm and fertilizin from the two other species tested (*Mellita quinquiesperforata* and *Echinarachnius parma*). Thus the fertilizin-agglutination inhibiting action of the *Fucus* extract was not specific.

The *Fucus* extract caused several visible changes in the sperm. In the highest concentrations of the most potent inhibitor preparations sperm slowly agglutinated when diluted in sea water. In more dilute (but still inhibiting) concentrations of the extract, the sperm became more motile than control sperm in sea water.

The inhibition of fertilizin agglutination of sperm could have resulted from an action of *Fucus* extract on fertilizin, on the sperm, or both. To test the first of these possibilities attempts were made to restore agglutinating activity to non-agglutinating *Fucus* extract-fertilizin mixtures (*cf.* Metz, 1959). As seen in Table I (one of four similar experiments) treatment with activated charcoal ("Norite") restored the agglutinating activity of such a mixture to virtually control levels. Apparently, the charcoal differentially adsorbed the inhibiting agent without appreciable effect on the fertilizin (*cf.* Tyler and Fox, 1940). It is clear from this that the *Fucus* extract does not irreversibly destroy the sperm agglutinating action of fertilizin. This view is supported also by the observation (Table I) that dilution of the non-agglutinating mixture with sea water restores agglutinating action. These observations suggest that the *Fucus* extract does not affect the fertilizin but instead acts upon the sperm.

To test more directly whether the inhibition resulted from an effect on sperm, the following experiment was performed. Sperm were mixed with *Fucus* extract to produce a suspension which did not agglutinate with fertilizin. Then the sperm were centrifuged down, the supernatant replaced with sea water and the sperm resuspended. This washing was repeated several times and the sperm tested for

Table I*

Agglutinating capacity of Arbacia fertilizin after exposure to Fucus extract

Dilution of mixture	Fertilizin + *Fucus* extract		Fertilizin + sea water	
	Absorbed with Norite	Unabsorbed	Absorbed with Norite	Unabsorbed
1/2	++++	−	++++	++++
1/4	++++	++	++++	++++
1/8	++++	++++	++++	++++
1/16	++++	++++	++	++++
1/32	+++	+++	+	+++
1/64	++	++	−	++
1/128	±	+	−	+

* One ml. of fertilizin was mixed with one ml. of *Fucus* extract (dialysed against sea water) or with sea water. One ml. of activated charcoal ("Norite") was then added to each and removed by filtration. The mixtures were tested for agglutinating action before and after absorption with charcoal. To test for agglutinating action one-drop aliquots of the filtrates were serially diluted in two-fold steps and mixed with one-drop samples of semen diluted to 1% with sea water. The results are recorded as agglutinations.

TABLE II*

Fertilization agglutination of Arbacia sperm after washing from Fucus extract

Times centrifuged	Sperm + *Fucus* extract centrifuged		Sperm + sea water centrifuged	
	Fertilizin agglutination of sperm after resuspension in sea water	Fertilizin agglutination of control sperm in supernatant	Fertilizin agglutination of sperm after resuspension in sea water	Fertilizin agglutination of control sperm in supernatant
1	–	–	++++	++++
2	++	++++	++++	++++
3	+++		++++	
4	++++		++++	

* Two-ml. samples of 5% semen were mixed with 2 ml. of *Fucus* extract (dialysed against sea water) or sea water. After 32 minutes both samples were centrifuged (4° C.), the supernatants were withdrawn and the sperm resuspended to 4 ml. in sea water. One-drop aliquots of sperm suspension were tested for agglutination by adding one drop of fertilizin. The centrifugation of the main sample was then repeated. One-drop aliquots of the supernatants were tested for excess inhibitor by adding one drop of 1% control sperm, then one drop of fertilizin. The results are recorded as agglutination.

agglutination with fertilizin after each washing. One of the six experiments performed appears in Table II. In all experiments the sperm failed to agglutinate after the first resuspension in the sea water, even though the wash water (the supernatant from the second centrifugation) did not inhibit when tested with control sperm. With repeated washing the spermatozoa regained some ability to agglutinate when mixed with fertilizin. The number of washings required to restore agglutinability to the sperm varied somewhat. In one experiment treated sperm did not agglutinate with fertilizin until after five washings. This experiment was run in parallel with fertilizin-treated reversed sperm, which also recovered agglutinability after five washings. Repeated washing altered the fertilizin

TABLE III*

Agglutination titer of fertilizin after exposure to Fucus extract-treated sperm.

Supernatant dilution	*Fucus* extract-treated sperm + fertilizin	Sea water-treated sperm + fertilizin	*Fucus* extract + fertilizin	Sea water + fertilizin
1/2	++++	++++	–	++++
1/4	++++	+++	–	++++
1/8	++++	++	–	++++
1/16	+++	+	+	++++
1/32	++	–	+	+++
1/64	+	–	+	++
1/128	–	–	+	+

* One ml. of packed sperm (or sea water) was mixed with one ml. of *Fucus* extract (or sea water) and centrifuged down (4° C.). The sperm (or one ml. of solution) were resuspended in two ml. of fertilizin and recentrifuged. One-drop aliquots of the supernatants were diluted in two-fold steps and mixed with one drop of 0.5% sperm. The results are recorded as agglutination.

TABLE IV*

Absorption of Fucus extract color and activity by Arbacia sperm

	Fucus extract + sea water	*Fucus* extract + sperm	Sea water + sperm	Sea water + sea water
O.D.	.86	.37	.06	.00
Fertilizin agglutination inhibition titer	6	0	0	0
Fertilization inhibition titer	8	2	0	0

* Five ml. of *Fucus* extract (1.0×10^{-4} gm./ml.) or sea water were mixed with two ml. of washed sperm or sea water and after five minutes centrifuged. Optical density of the resulting supernatant was measured with a Klett-Summerson colorimeter (420 μm.). Fertilizin agglutination inhibition titer is the reciprocal of the last dilution (in a two-fold dilution series) in which no agglutination occurred when one drop of inhibitor was mixed with one drop of 0.5% semen. Fertilization inhibition titer is the reciprocal of the last dilution (two-fold) in which 1% or less cleavage occurred after one drop of eggs was added and inseminated with two drops of 10^{-3} semen.

agglutination of sperm. The agglutinates formed more slowly than with unwashed sperm, and also reversed more slowly, even though the spermatozoa remained motile. This effect was more pronounced on the inhibitor-treated sperm than on sperm treated in sea water.

The above experiments indicate that the *Fucus* extract acted upon the sperm to inhibit agglutinability with fertilizin. Additional evidence for this was found in experiments demonstrating that the inhibitor-treated, non-agglutinating sperm failed to absorb fertilizin as readily as did control sperm (Table III) (*cf.* Lillie, 1914). In two experiments inhibitor-treated and control sperm were added to fertilizin, centrifuged out, and the supernatant titrated for agglutinating activity. In both experiments the control sperm reduced the agglutinating activity substantially more than inhibitor-treated sperm. It is concluded from these experiments that the *Fucus* extract inactivated the specific fertilizin receptor sites of the sperm surface (antifertilizin) to render them unreactive to fertilizin. "Antifertilizin" extracted from sperm by the freeze-thaw method of Tyler (1939) lost its egg jelly coat precipitating activity after mixing with *Fucus* extracts, although dilution controls remained active. A precipitate formed when the "antifertilizin" preparation was mixed with *Fucus* extract.

Sperm appeared to remove the brown color from *Fucus* extract solution. This was confirmed by measuring the optical density of a *Fucus* extract solution with a Klett-Summerson colorimeter (blue filter, 420 mμ.) before and after absorption with sperm (Table IV). The solution was also titrated for inhibitory activity after absorption with sperm. In five experiments sperm decreased the optical density of *Fucus* extract solutions and removed or greatly reduced both the fertilizin agglutination and the fertilization inhibiting properties of the solutions. The sperm used in the absorption became more darkly colored than control sperm. Thus, it appears that sperm reacted with and removed from solution the color and active substance(s) of the *Fucus* extract, and that concomitantly the sperm lost the ability to react with fertilizin from the egg.

Sperm motility and oxygen consumption in Fucus extracts

Wicklund (1954c) reported that sperm were more active in FeInh(Fu) than in sea water. The extracts prepared in this laboratory also stimulated the motility of spermatozoa. However, in high concentrations of the more potent inhibiting preparations, sperm became immotile more quickly than sperm in sea water.

The stimulation of sperm was also examined in terms of oxygen consumption measured manometrically with the Warburg apparatus. In four experiments the rate of oxygen consumption increased dramatically upon the addition of *Fucus* extract to the sperm (Fig. 2). In the experiments represented in the figure the

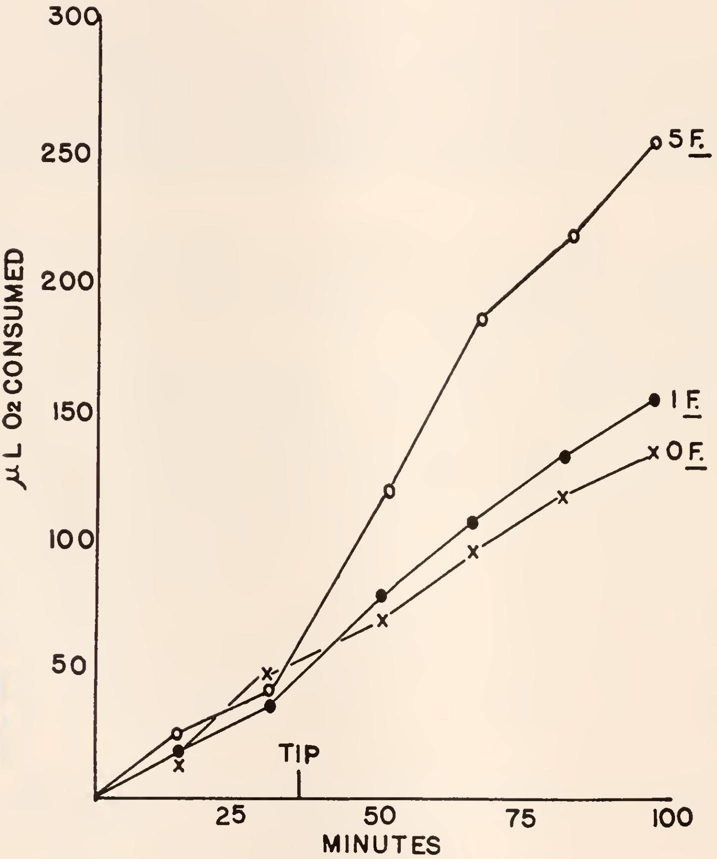

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FIGURE 2. Effects of *Fucus* extracts on the oxygen consumption of sperm. Curve 5F (open circles) 5.0×10^{-4} gm. of Fucus extract per ml.; Curve 1F (solid circles) 1.0×10^{-4} gm. of *Fucus* extract per ml.; 0F (crosses) sea water control containing no *Fucus* extract. Oxygen consumption was measured with a standard Warburg apparatus. Experiments were performed in duplicate in 15–20-ml. single side arm flasks at 20° C. The vessels were shaken at 120 cycles/min, with an amplitude of 3.5 cm. Vessels contained 1.8 ml. of washed sperm (2.0×10^{10} sperm/ml.). Two-tenths ml. of 10% KOH was in the center well. One ml. of sea water or *Fucus* extract was tipped into the vessels at 35 minutes. All solutions were buffered to pH 8.0 with 0.02 *M* glycylglycine.

pH was controlled by using sea water buffered with .02 *M* glycylglycine (Tyler and Horowitz, 1937). At the beginning of the experiment sea water, sperm and *Fucus* extract were adjusted to pH 8.0. At the end of the experiment, one hour after tipping the test substance, the pH was again measured and had dropped to pH 7.8 in the most actively respiring systems.

The observed increase in oxygen consumption was clearly the result of enhanced sperm respiration, not a direct oxidation of the *Fucus* extract. This was shown in three experiments in which *Fucus* extract did not consume oxygen when tipped into sea water.

It will be seen from Figure 2 that the rate of O_2 consumption increased upon addition of *Fucus* extract. The rate was maximum directly after tipping and then decreased. No dilution effect was observed in the sea water control. The change in rate of oxygen consumption was related to the concentration of the *Fucus* extract. A maximum rate (0–15 minutes after tipping) of 6.4×10^{-9} μl. O_2/hr./sperm resulted when 5.0×10^{-4} gm. of extract was added to 3.6×10^{10} sperm (or 1.4×10^{-14} gm./sperm). This represents a 2.8-fold increase over the control rate of 2.3×10^{-9} μl. O_2/hr./sperm. When 1.0×10^{-4} gm. of extract was added to the same amount of sperm (or 0.28×10^{-14} gm./sperm) only a 1.3-fold increase in rate was observed (to 3.1×10^{-9} μl. O_2/hr./sperm). In a separate experiment, employing a one-tenth dilution of the same sperm used in the experiment in Figure 2 (3.4×10^{9} sperm), the addition of 1.0×10^{-4} gm. of extract (or 2.9×10^{-14} gm./sperm) resulted in a 6.2-fold increase in rate (from 8.2×10^{-9} to 51×10^{-9} μl. O_2/hr./sperm).

Fertilizing capacity of Fucus extract-treated sperm

Inhibition of fertilization apparently does not result from an irreversible action on spermatozoa. Wicklund (1954a) reported that sperm suspended in FeInh(Fu) were capable of fertilizing eggs if the inhibitor was diluted out during insemination. The *Fucus* extracts prepared in this laboratory were tested for effects on the fertilizing capacity of *Arbacia* sperm. The results obtained were basically the same as reported by Wicklund. That is, treated sperm were capable of fertilizing eggs. However, in 8 of 22 experiments the fertilizing capacity of treated sperm was reduced, in the sense that more treated than control sperm were required to achieve the same percentage of cleaved eggs.

To test for effects on the fertilizing capacity, *Arbacia* sperm were suspended in *Fucus* extract solution, then centrifuged down and resuspended in sea water. The washed sperm were then serially diluted and used to inseminate eggs. The supernatant removed after centrifugation was tested for an excess of fertilization inhibitor by adding eggs and inseminating with control sperm. The agglutinating capacity of the treated sperm was tested by adding a drop of fertilizin to a drop of washed sperm. Only those experiments wherein the treated sperm failed to agglutinate are considered. The number of sperm was determined turbidimetrically after washing to be sure that the amounts of treated and control sperm were equal.

In 14 experiments sperm treated in *Fucus* extracts were as successful in activating eggs as sperm treated in sea water. In 8 experiments, however, more *Fucus* extracted-treated sperm than control sperm were required to achieve equivalent percentages of cleaved eggs. One of these experiments is presented in Table V.

TABLE V*

The fertilizing capacity of Fucus extract-treated sperm

Sperm/ml.	*Fucus* extract-treated sperm 4 ml. + 1 drop eggs	Sea water-treated sperm 4 ml. + 1 drop eggs
6.4×10^9	100% cleaved	100% cleaved
1.3×10^9	99%	100%
2.6×10^8	9%	86%
5.4×10^7	5%	55%
1.1×10^7	1%	2%
2.2×10^6	0	1%
Inhibitor solution after centrifugation	0	100%

* Two ml. of diluted *Arbacia* sperm were mixed with two ml. of *Fucus* extract (2.5×10^{-5} gm./ml.) or with sea water. The suspension was centrifuged in the cold and the sperm resuspended in 10 ml. of sea water. The sperm concentration was determined turbidimetrically and the suspension diluted in five-fold steps. One drop of eggs was added to four ml. of the sperm. Eggs were also added to the supernatant (solution after treatment of sperm) and inseminated with one drop of 3.2×10^{10} sperm/ml. Results are presented as percentage of cleaved eggs (100 eggs counted).

In all eight of the experiments wherein reduced fertilizing capacity was observed, sperm were treated with samples from a single very "active" inhibitor preparation (100% inhibition of cleavage in 1.88×10^{-6} gm. of *Fucus* substance/ml.). The highest percentage of cleavage obtained in each of these eight experiments occurred in the highest concentrations of sperm and consequently the highest concentrations of any residual inhibitor. In four experiments the same inhibitor did not reduce the fertilizing capacity of sperm, although an excess of inhibitor was demonstrated in the first supernatant (treating solution) and the treated sperm failed to agglutinate when added to fertilizin solution.

Fertilizability of Fucus extract-treated eggs

Wicklund (1954a) reported that the effect of FeInh(Fu) on fertilization was reversible. Eggs treated with the *Fucus* extracts prepared in this laboratory, however, failed to recover fertilizability after repeated washing with sea water, or digestion by enzymes. This inhibition was not the result of "killing" the eggs, for eggs fertilized in sea water and then placed in otherwise inhibitory solutions of *Fucus* extracts cleaved normally.

Eggs were exposed to *Fucus* extract solution, extensively washed in sea water and then inseminated. The results of one of nine similar experiments are presented in Table VI. It is clearly seen that eggs treated with the extracts are not fertilizable even after repeated washing with sea water. In the nine experiments some of the eggs were also passed through (one hour) 1% solutions of trypsin, lysozyme, pectinase or pectinesterase. Control eggs remained fertilizable after exposure to the protein solutions but eggs exposed to *Fucus* extract, before digestion with the above enzymes, failed to cleave after insemination.

This irreversible inhibition of fertilization is apparently not due to general cytotoxic effects on eggs. Eggs placed in fertilization-inhibiting concentrations of

TABLE VI*

Fertilizability of Fucus extract-treated eggs

Sperm dilution	*Fucus* extract-treated eggs washed in sea water	Sea water-treated eggs washed in sea water
1	0 cleaved	95% cleaved
1/5	0	95%
1/25	0	100%
1/125	0	92%
1/625	0	63%

* Ten drops of *Arbacia* eggs were placed in five ml. of sea water or *Fucus* extract ($2.5 \times 10^{-}$ gm./ml.) for five minutes. The eggs were then washed through 8 five-ml. changes of sea water over a period of 90 minutes. One drop of eggs was added to four ml. of serially diluted sperm and the results reported as per cent cleavage (100 eggs counted).

extract five minutes after insemination cleaved synchronously with untreated controls if left in the inhibitor or washed out of it. Also, in three experiments eggs predigested with trypsin were not inhibited by exposure to *Fucus* extract. These experiments indicate that the *Fucus* extract solutions do not kill eggs or inhibit the cleavage process, but do interfere with or block an initial stage(s) of fertilization.

Effects of tannic acid on Arbacia gametes

Similarities between the effects of *Fucus* extracts and tannic acid on *in vitro* enzyme systems were pointed out by Esping (1957b). Kylin (1938) found that brown algae contained substances similar to tannins. Thus it seemed of interest to examine the effects of tannic acid on sea urchin gametes and fertilization (*cf.* Branham and Metz, 1960). It was found that tannic acid affected sperm in a manner similar to *Fucus* extracts and inhibited fertilization by an irreversible action on eggs.

Baker (C.P.) tannic acid was found to inhibit fertilizin agglutination of sperm (*Arbacia*) in concentrations of about 3.2×10^{-3} gm./ml. Sperm remained motile at this dilution. In three experiments sperm treated with tannic acid, centrifuged down and resuspended in sea water failed to agglutinate when tested with fertilizin, even though the supernatant from a second centrifugation (the wash water) was free of inhibitory activity when tested on control sperm. Repeated washing restored some agglutinability to the treated sperm but the results of these experiments were equivocal in that such repeatedly washed sperm spontaneously agglutinated upon dilution in sea water.

Tannic acid also inhibited fertilization. Complete inhibition of cleavage resulted when eggs were inseminated in concentrations of 6.0×10^{-5} gm. of tannic acid/ml. or greater. Washing tannic acid-treated eggs in sea water failed to restore fertilizability. The motility of sperm was not impaired in inhibiting concentrations of tannic acid, and eggs placed in the inhibiting solution 5 minutes after insemination cleaved normally. Thus tannic acid and *Fucus* extracts both inhibit fertilization by an irreversible action on eggs.

It was also found that the inhibitory titer of tannic acid increased upon oxidation. In one experiment 1% tannic acid in distilled water was raised to pH 10 with NaOH and air was bubbled through the solution for one week. The preparation was then dialysed against distilled water and tested for inhibitory effects. This treatment increased the capacity of tannic acid to inhibit fertilizin agglutination of sperm by about 10-fold (from 3.2×10^{-3} gm./ml. to 2.2×10^{-4} gm./ml. for complete inhibition), but the fertilization inhibition titer remained essentially the same as before the oxidation.

Discussion

The experiments presented here demonstrate several effects of extracts of the brown alga *Fucus vesiculosus* upon sea urchin gametes. These include stimulation of motility and increase in rate of oxygen consumption of spermatozoa, inhibition of agglutination of sperm by fertilizin and finally inhibition of fertilization.

The inhibition of fertilizin agglutination of sperm results from the action of the inhibiting preparation on the sperm to render these cells unagglutinable to fertilizin. Evidence for such action was obtained in washing experiments. Sperm treated with inhibiting concentrations of *Fucus* extract failed to regain agglutinability upon washing in sea water. Repeated washings, however, do restore agglutinability to treated sperm. To this extent the effect of the inhibitor is reversible. The *Fucus* extracts not only inhibited agglutination but actually rendered sperm incapable of combining with fertilizin. This action was demonstrated by failure of treated sperm to absorb fertilizin. The *Fucus* preparation failed to irreversibly inactivate fertilizin, as shown by restoration of agglutinating activity to non-agglutinating fertilizin-*Fucus* extract mixtures by absorption with charcoal.

One explanation for the observed inhibition of fertilizin agglutination is that inhibitory material in the *Fucus* extracts combined with the sperm surface to inactivate the antifertilizin. The observation that sperm removed the inhibitor from solution is additional evidence for such a mechanism. The inactivation could result from direct combination of the inhibitor with the active site or from a steric masking of the antifertilizin. The recovery of agglutinability upon repeated washing can be explained by removal of the masking substance or by stripping off of the inactivated antifertilizin-inhibitor complex to expose unreacted sites.

Another explanation is that the inhibitor removed antifertilizin from the sperm surface. The gradual recovery of agglutinability with repeated washing is evidence that this is not the case. If it is assumed that the recovered agglutinability is due to the same kind of reactive site as normal agglutination, it would be expected that the effect of the inhibitor would disappear as soon as the sperm were washed. The observed slow appearance of agglutinability after repeated washing is not accounted for by this hypothesis.

Esping (1957b) suggested that *Fucus* extracts denature proteins in the same way as tannic acid. The observation of Wicklund (1954a) that albumin and some other proteins reverse the effect of FeInh(Fu) on eggs could also be explained by a tanning reaction between the inhibitor and the protein resulting in inactivation or removal of the inhibitor. The similarities reported here between the action of *Fucus* extracts and tannic acid suggest that the inhibitory effects might be due to a tanning action. The inhibition of fertilizin agglutination could be explained by hypothesizing precipitation of sperm surface proteins by depsides (aromatic tannagins).

If *Fucus* extracts are assumed to contain depside-like characteristics, then the failure of the inhibitor to destroy the agglutinating properties of fertilizin is of some interest. Fertilizin apparently is an acid mucopolysaccharide which is not readily separated into distinct protein and carbohydrate moieties (Metz, 1957). At least one way that depsides react with proteins is through amino groups of the protein by hydrogen bonding or ionic linkage with anionic sites on the tannic acid (Gustavson, 1956). Failure of tannic acid to inactivate fertilizin, then, indicates that such cationic groups are not essential for agglutinating activity. This conclusion agrees with results obtained previously using protein group reagents (Metz, 1954).

The stimulation of motility and the increased rate of O_2 consumption that occurs when spermatozoa are mixed with *Fucus* extracts are striking but shed little light on the mechanism of inhibitor action. Numerous agents cause similar effects (*cf.* Rothschild, 1956). In experiments with *Fucus* extracts, sperm at first become hyperactive but later immotile. The rate of respiration remained high even after the sperm became immotile. This suggests that the *Fucus* substances could uncouple oxidative phosphorylation.

Despite the effects of *Fucus* extracts on fertilizin agglutination and respiration, sperm treated with the extracts are capable of fertilizing eggs. In 14 of 22 experiments the fertilizing capacity of treated sperm was the same as that of control sperm, even though the treated sperm did not agglutinate with fertilizin. This observation is in agreement with the report of Tyler and Metz (1955) that fertilizin-treated *Arbacia* sperm showed no reduction of fertilizing capacity when tested on normal eggs. These experiments also indicate that the *Fucus* extracts had no deleterious effects upon the egg-activating properties of spermatozoa. Thus sperm motility, acrosome reaction and surface groups that can be postulated to have a role in egg activation must not have been impaired by exposure to the fertilization inhibitor from *Fucus* in these 14 experiments. In 8 other experiments the fertilizing capacity of sperm was reduced upon exposure to *Fucus* extracts. The nature of this reduction has not been explained but it is interesting that it was not absolute. If enough treated sperm were present, eggs were activated. This indicates either that many partially inhibited sperm could combine forces to bring about egg activation or that some sperm were not inactivated at all. The all-or-none hypothesis seems more reasonable because the resulting cleavages were normal, that is to say, into two equal blastomeres, indicating reaction with a single spermatozoan. In all 22 experiments the treated sperm were capable of fertilizing eggs, and thus an explanation of the action of the fertilization inhibitor from *Fucus* must be sought elsewhere.

The inhibition of fertilization results from an irreversible action upon eggs. This is clearly demonstrated in numerous experiments wherein eggs were washed repeatedly in sea water following treatment in *Fucus* extracts but remained unfertilizable. This irreversible inhibition apparently does not involve general cytotoxic effects upon eggs. This is demonstrated by the normal cleavage of eggs inseminated in sea water and then placed in otherwise inhibiting concentration of the *Fucus* extracts. Eggs pretreated with trypsin and subsequently exposed to the extracts, fertilized upon insemination, again demonstrating that the *Fucus* extracts apparently have no general toxic action on the egg. Fertilization inhibition apparently involves inactivation of some initial step(s) of fertilization of the egg, perhaps by a depside-like reaction with the egg surface. In further studies attempts will be made to identify the site of such action.

SUMMARY

1. Extracts of the brown alga *Fucus vesiculosus* inhibit fertilization and the fertilizin agglutination of *Arbacia* sperm.

2. The inhibition of fertilizin agglutination apparently results from an action on sperm. Sperm washed from the extracts do not agglutinate. Furthermore, fertilizin is not irreversibly inactivated by *Fucus* extracts. The color and inhibitory properties of *Fucus* extracts are removed from solution by absorption with sperm.

3. Sperm motility and O_2 consumption were stimulated by *Fucus* extracts.

4. The inhibition of fertilization is the result of an irreversible action on eggs. The fertilizing capacity of sperm, however, is not destroyed by exposure to inhibitory amounts of *Fucus* extract.

5. Tannic acid showed inhibitory properties similar to the *Fucus* extracts. It inhibited fertilization by an irreversible effect on eggs and prevented fertilizin agglutination by an effect on sperm. Tannic acid-treated sperm were capable of fertilizing eggs.

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ASPECTS OF OSMOREGULATION IN TWO SPECIES OF INTERTIDAL CRABS[1]

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Considerable attention has been given problems of osmotic behavior in decapod Crustacea. Much of this work has concerned intertidal and semi-terrestrial species. Less information is available for species inhabiting estuarine conditions. Krogh (1939) and Prosser and Brown (1961) have reviewed extensively the subject of osmotic and ionic regulation. In the last several years Gross (1955, 1957a, 1957b, 1958, 1959) has contributed significantly to the understanding of water and ion balance in several species of crabs, with particular emphasis on *Pachygrapsus crassipes.*

For the past few years efforts have been made to understand aspects of the physiology of *Hemigrapsus oregonensis* and *H. nudus* (Dehnel, 1960). This previous work has shown that oxygen consumption measurements for animals maintained at a constant acclimation temperature, but a series of acclimation salinities, are higher at the lower salinities. This work has led to the suggestion that this increased respiratory rate at low salinities is the result of increased osmotic work, and not the result of increased muscular activity. Increased oxygen consumption has been demonstrated for crabs kept in an osmotic stress (Schlieper, 1929; Schwabe, 1933; Flemister and Flemister, 1951). These workers have interpreted their data to mean that increased oxygen consumption reflects increased osmotic work. Krogh (1939) and Wikgren (1953) tend to refute this idea. Gross (1957a) presents evidence also to the contrary, interpreting the results in terms of increased muscular activity.

It seems reasonable that both ideas are valid, depending upon the animal in question. *Pachygrapsus crassipes,* for instance, probably never encounters hypotonic environmental conditions. Both species of *Hemigrapsus* in this geographic region are exposed always to low salinity conditions during the summer months, in conjunction with relatively high temperatures. The reverse is true during the winter, although the sea water salinity is lower than 100%. Because of seasonal variation in salinity it would appear quite probable that *Hemigrapsus* would be a strong hyper-osmotic regulator, and as the osmotic gradient increased between blood and sea water, additional work would be necessary in order to maintain the gradient. This might be reflected in the oxygen consumption of the whole animal. The problem is to detect increased metabolic work, by measuring oxygen consumption, and to relate it to increased osmotic work. As yet, this relationship has not been resolved.

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The present study has determined the osmotic behavior of the two species of *Hemigrapsus* when exposed to a series of salinities, as well as to changing seasonal field salinities. Further, the effect of temperature on osmotic regulation was investigated, by exposing crabs to various combinations of temperature and salinity.

Material and Methods

Both species of crabs used for this study were collected from Spanish Bank, Vancouver, British Columbia. Experiments were conducted at a series of temperatures and salinities on animals collected from summer and winter populations. Seasonal field variation in these two environmental parameters has been described (Dehnel, 1960). Winter animals were returned to the laboratory and placed directly into an experimental temperature, but at a holding salinity of 75% sea water for 18 hours maximum. Crabs were transferred then to the experimental salinity and temperature conditions. Summer animals followed the same sequence except that these animals were maintained at the holding salinity for approximately 30 hours. Winter and summer animals were placed initially in 75% sea water to permit a common salinity facilitating comparison for all experimental series. Animals were placed directly into the experimental temperatures except for 5° and 25° C.; these were placed in room temperature water (16° to 18° C.) for approximately three hours, then into the controlled-temperature refrigerators. Throughout the series of experiments crabs were kept in the dark and not fed. Crabs were maintained in plastic containers, approximately 30 animals per container. Water of the appropriate salinity and temperature was changed daily. Crabs were fully immersed during the experimental periods.

Experimental temperatures used were 5°, 10°, 15°, 20° and 25° C. (± 1.0° C.) and experimental salinities were 6, 12, 25, 75, 100, 125, 150 and 175% sea water. Salinities above 100% sea water were obtained by adding appropriate amounts of reagent sodium chloride. Salinities below 100% were obtained by adding distilled water to normal sea water. All field and experimental salinities are expressed as percentage sea water, based on a standard sea water, 31.88‰ salinity, 17.65‰ chlorinity at 25° C. as 100% sea water. Salinities were determined on a 1000-cycle conductivity bridge calibrated to the standard sea water noted above.

Melting points of sea water concentrated by several methods were determined to establish whether total osmotic pressure differed. Boiling and freezing low salinity water to 100% sea water resulted in essentially the same value. Sea water concentrated to 150% by addition of NaCl, then diluted to 100% with distilled water gave a somewhat higher but statistically insignificant value when compared with the other two methods. Similarity of melting point values would suggest that the ion balance of concentrated sea water was not significantly altered by addition solely of NaCl.

Blood was sampled from the crabs by two methods: removal of the coxopodite of the last pereiopod, or puncture of the membrane proximal to the coxopodite. The area was damp-dried and blood was allowed to flow from the region of removal or puncture. This area was dried again and then the blood was sampled. The sample was collected in a blood capillary tube (0.4 mm. I.D.), sealed with Nevastane grease and frozen on dry ice. Puncture of the membrane permitted the animal to be returned to the experimental conditions for further sampling. Animals from which a leg was removed were discarded. The two sampling techniques produced identical results, as the puncture closed rapidly. The puncture technique was used on summer animals to facilitate judicious use of the crab population. At the time of each collection, blood from a group of animals was sampled immediately upon return to the laboratory. The blood concentrations of these animals provided data which demonstrated periodic blood changes, due to variations of field salinity. Field temperature and salinity data were obtained for each collection. Blood of crabs held at the experimental conditions was sampled at three, 24 and 48 hours following placement into the experimental conditions. A modified method for melting point determination, as described by Gross (1954), was used to determine total osmotic pressure. Ten to 15 animals were sampled to determine osmotic pressure of the blood at each time period. Points on the various graphs represent a mean of the measurements for each period.

For all experimental series a weight range was selected. Weight of summer animals ranged from approximately 1.0 gram to 5.0 grams; winter animals, from 0.5 gram to 5.0 grams. During the winter a parallel series of experiments was conducted to determine whether a change in total body weight could be detected in hypo- and hypertonic media. Crabs were damp-dried and weighed before and after exposure to the experimental conditions. Further, dry weights were determined for groups of crabs removed directly from field conditions, and for ones after exposure to the experimental conditions.

In Figures 1, 2, 5 and 6, per cent sea water of the body fluid of each species (summer and winter) at time zero was based on the mean of all three interval readings at 75% sea water for all temperatures. The difference between summer and winter groups of each species was small.

The term "gradient" is used to indicate the difference in concentration (expressed in per cent sea water) between the blood and the external medium. The data permitted use of the Student's "t" test for statistical treatment. Significance is considered at the 0.01 level of probability.

RESULTS

Hemigrapsus oregonensis

Response to external salinity changes

The change in blood concentration as effected by different external sea water concentrations is shown in Figures 1 (summer) and 2 (winter). Results for summer animals were determined at 15° C. and those for the winter, at 5° C. These two temperatures approximate field temperature conditions for the two seasons. In general, changes in blood concentration were rapid; approximately one-half of the total change occurred within the first three hours. At the end of 24 hours, total blood concentration changes for summer animals (Fig. 1) had occurred in 100% to 6% sea water, and were hypertonic to these external media. After a 48-hour period, changes in the blood of animals in 125% and 150% sea water were not complete, but all were hypertonic to the external sea water. Crabs kept in 175% sea water died, following the three-hour period of exposure. The results for winter animals (Fig. 2) showed the blood to be hypertonic at all external

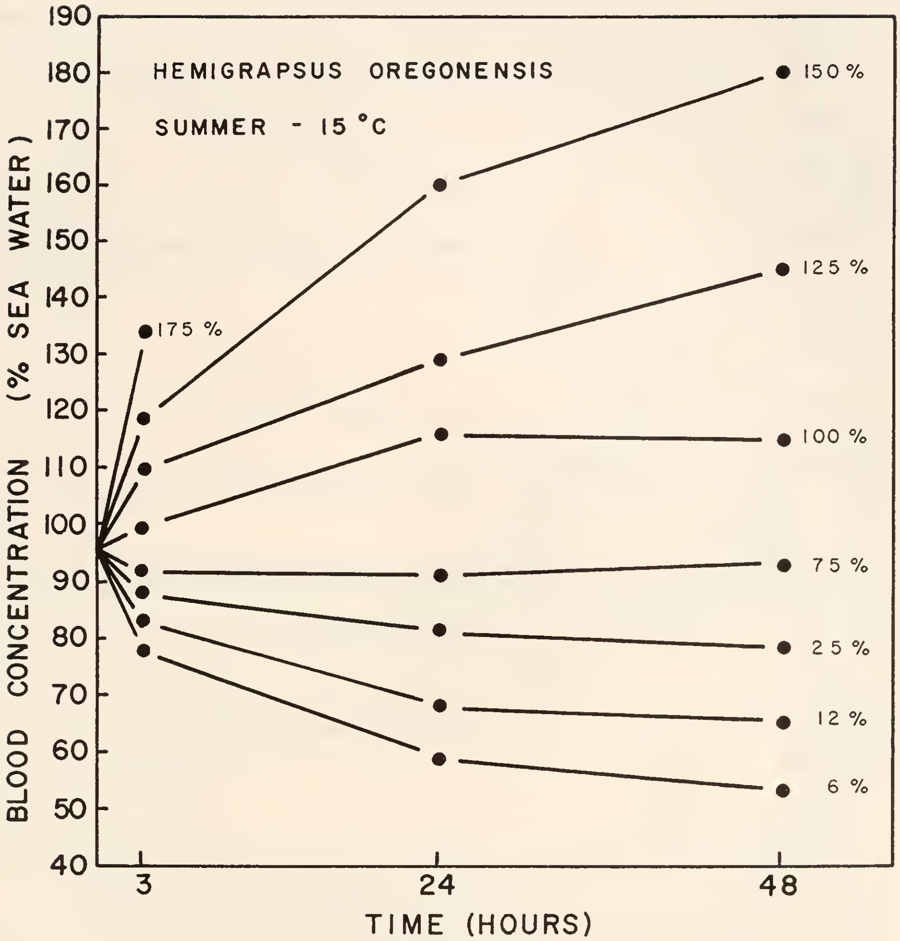

FIGURE 1. Osmotic regulation in summer *Hemigrapsus oregonensis,* at 15° C., as a function of time in the experimental salinities. Each point represents the mean of the measurements of 10 to 15 animals for each time period. Sea water concentrations are indicated in per cent normal sea water for each of the respective curves.

salinities, reaching a steady state after 24 hours' exposure, except 175% sea water. At this concentration the blood was still changing after the 48-hour period of exposure. It was assumed, however, that all major changes in blood concentration had occurred at the end of 48 hours.

Seasonal effect of temperature

The effect of temperature (5°, 15° and 25° C.) on the blood concentration for summer and winter animals exposed to different salinities is given in Figure 3. These results showed that blood concentrations were hypertonic to all external salinities at all temperatures. Data for 10° and 20° C. (not presented) are further documentation. At salinities less than 100% sea water, blood concentration of

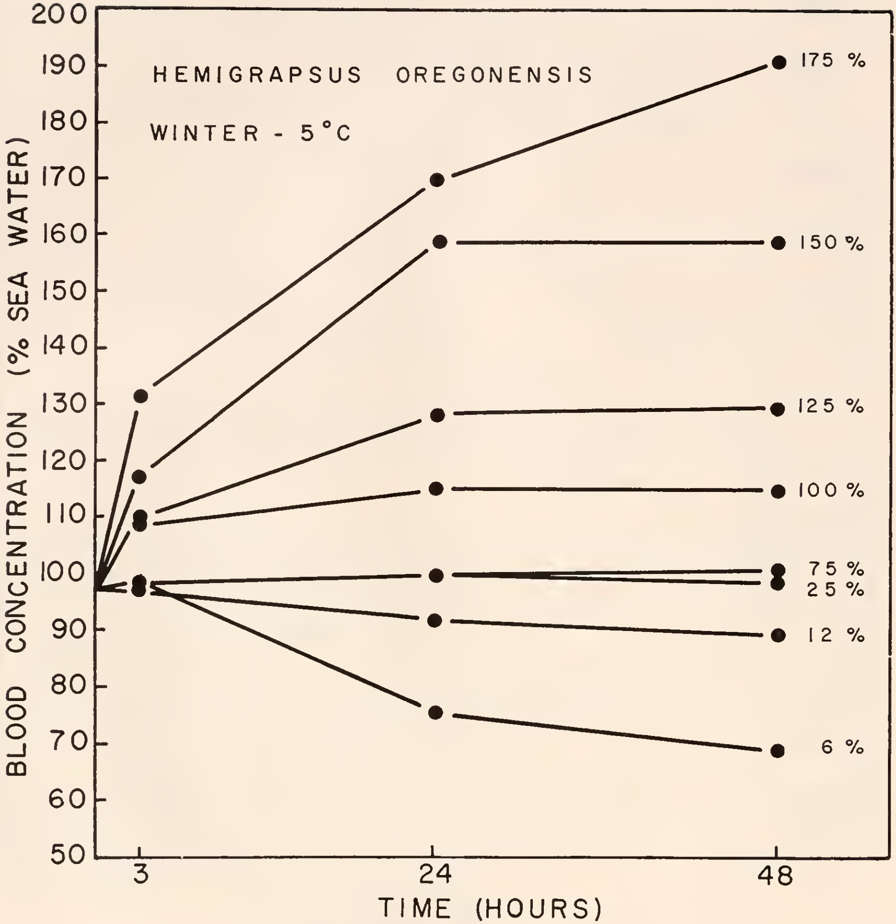

FIGURE 2. Osmotic regulation in winter *Hemigrapsus oregonensis*, at 5° C., as a function of time in the experimental salinities. Each point represents the mean of the measurements of 10 to 15 animals for each time period. Sea water concentrations are indicated in per cent normal sea water for each of the respective curves.

winter animals at the two lower temperatures was higher than that of summer animals at any of the temperatures ($P = 0.01$). At 100% sea water, blood concentrations for summer and winter animals for all temperatures were similar. Above 100% sea water the blood concentration for summer animals at 5° and 15° C. was significantly higher ($P = 0.01$), when compared with winter crabs.

Comparison of blood concentration of summer animals at all temperatures and at each salinity showed some difference (Fig. 3). For any salinity, the effect produced by the maximum temperature difference (20° C.) was of the order of 10%. When the summer 5° C. and 25° C. curves are compared at various salinities, the differences are statistically significant at 6, 100 and 125% sea water ($P = 0.01$). The same comparison for winter animals gave a somewhat greater

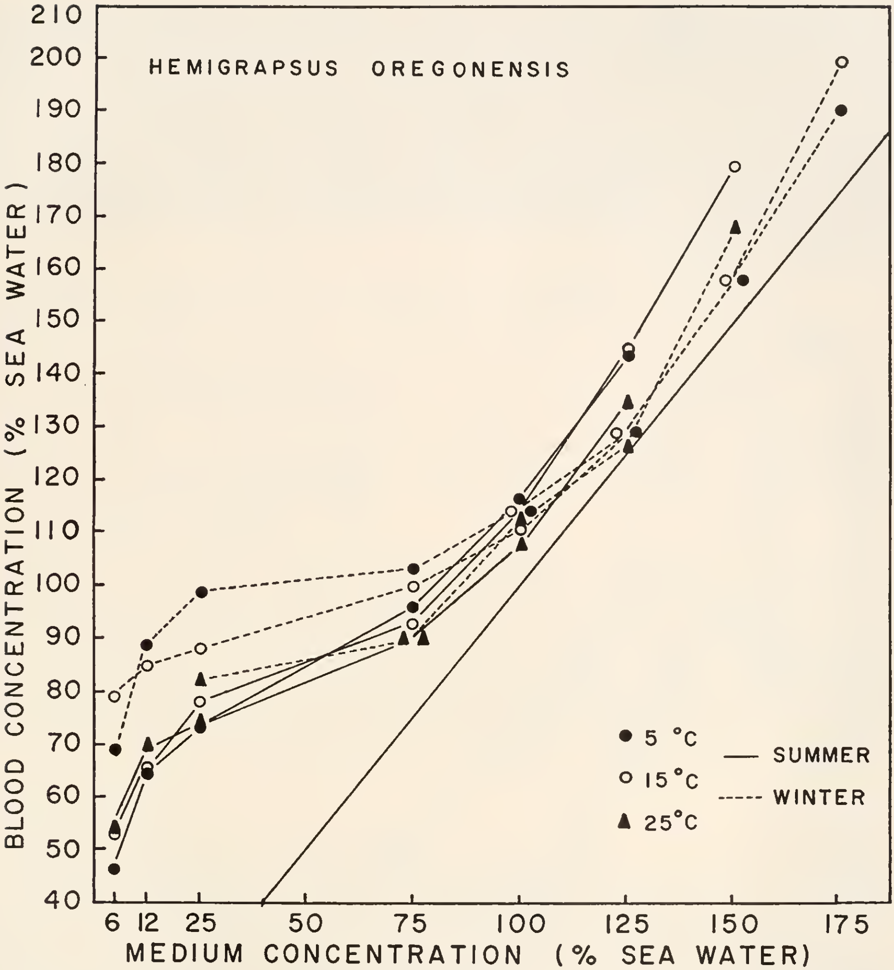

FIGURE 3. Relationship of the body fluid concentration of summer and winter *Hemigrapsus oregonensis,* at 5°, 15° and 25° C., to the medium concentrations after exposure for 48 hours to the experimental salinities.

concentration difference at salinities below 100% sea water. For example, at 25% sea water, the absolute difference between 5° and 25° C. was 17% ($P = 0.01$). As external salinity increased, the difference in blood concentration between low and high temperatures decreased, and a minimum was reached between 100% and 125% sea water for both summer and winter crabs. Above this concentration the difference increased slightly. It should be noted that at the lower salinities (12% to 75% sea water) the highest blood concentration, for winter animals in particular, was found generally at the lowest temperature (5° C.), *i.e.,* as the concentration of the external sea water decreased, blood concentration increased as temperature decreased.

When winter animals (Fig. 3) at their own approximate temperature and salinity field conditions (5° C.; 75% sea water) were compared with summer crabs at their corresponding conditions (15° C.; 25% sea water), blood concentration of winter crabs was more hypertonic (25% sea water difference; $P = 0.01$). When the blood of winter crabs was tested immediately upon removal from the intertidal zone, its concentration was always higher than that of summer crabs similarly removed. Further, comparison at either season showed the blood concentration always to be higher than the intertidal sea water concentration from which they were removed.

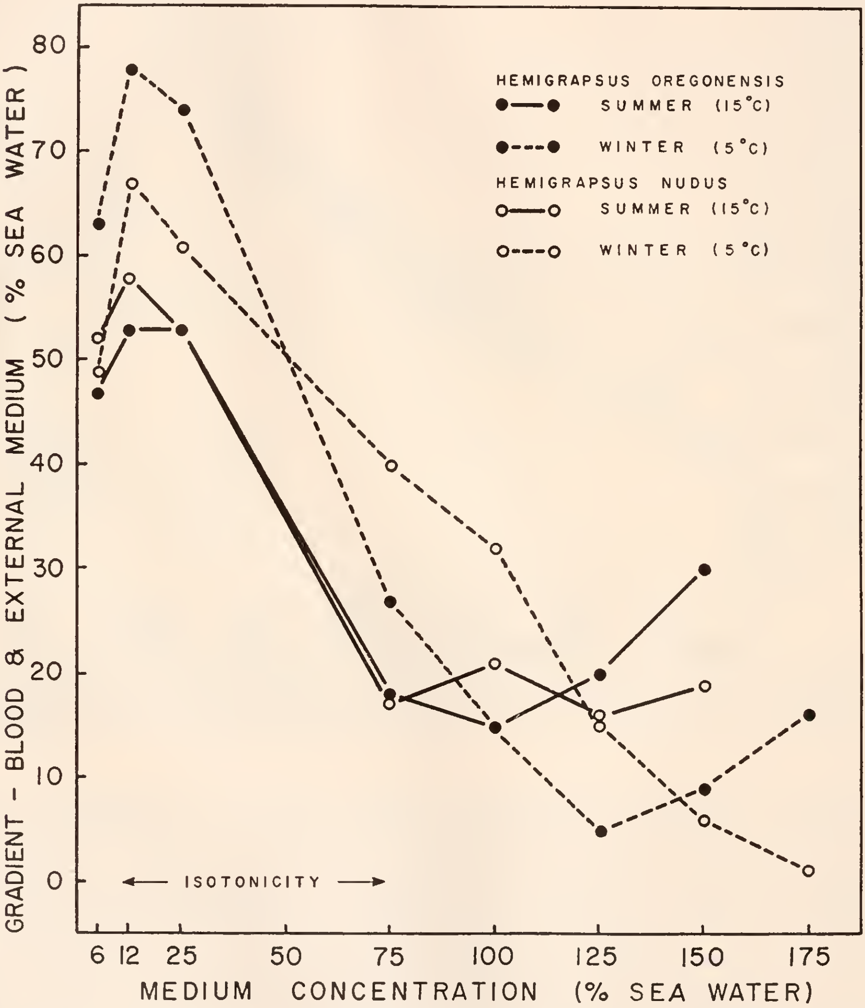

FIGURE 4. Relationship of the gradient between blood and external sea water to medium concentrations in summer (15° C.) and winter (5° C.) *Hemigrapsus oregonensis* and *Hemigrapsus nudus*, after exposure for 48 hours to the experimental salinities.

Figure 4 compares the concentration gradients between blood and sea water for summer and winter *H. oregonensis* at their approximate seasonal intertidal temperatures. These data showed that winter crabs maintained a greater gradient ($P = 0.01$) at all sea water concentrations below 100%. Above 100% sea water summer crabs maintained the greater gradient ($P = 0.01$). At 100% sea water summer crabs showed the greatest reduction in gradient, whereas winter animals approximated isotonicity at 125% sea water. Both groups increased steadily with increasing concentration of the external medium, but summer crabs had the greater absolute increase. The largest gradient between blood and external medium for summer and winter animals was at 12% sea water. For instance, winter *H. oregonensis* at 12% sea water maintained a 78% gradient, summer crabs, a 53% gradient, a statistically significant difference of 25%. Winter crabs are better regulators in hypotonic media and summer crabs are better regulators in hypertonic media. The differences at each salinity between summer (15° C.) and winter (5° C.) crabs are significant ($P = 0.01$) except at 100% sea water.

Data in Figure 3 show blood concentrations of summer and winter animals hypertonic to all external salinities. These blood concentrations, when compared with the respective external salinity, were significant, except for winter animals at 25° C. and 125% sea water.

Mortality occurred at some of the temperature and salinity combinations. Generally, at the higher salinities (150% and 175% sea water) summer animals died at the higher experimental temperatures (15° and 25° C.) during the 24-hour period of exposure. At 5° and 10° C. crabs survived the first day but died during the second day. Winter *H. oregonensis* were more resistant, and deaths occurred only at higher temperatures (20° and 25° C.) when exposed to the highest and lowest salinities, and only then after the 24-hour exposure period.

Hemigrapsus nudus

Response to external salinity changes

Blood concentration changes for summer (15° C.) and winter (5° C.) populations of this species, as effected by different external salinities, are presented in Figures 5 (summer) and 6 (winter). Major changes were rapid as in *H. oregonensis,* and occurred within three hours. After 24 hours, the maximum change for summer crabs essentially was reached (Fig. 5), and the blood of these crabs was hypertonic to all experimental salinities. Crabs exposed to 175% sea water died after three hours. Winter crabs showed less initial change and following 24 hours of exposure, changes still were evident (Fig. 6). Concentration of blood of winter crabs was hypertonic to all external media, but at 150% and 175% sea water blood approached isotonicity.

Seasonal effect of temperature

Seasonal temperature effect (5°, 15° and 25° C.) on the blood concentration of summer and winter *H. nudus* when exposed to different external salinities is shown in Figure 7. Blood concentrations were hypertonic at all external salinities and temperatures. However, at 150% and 175% sea water, blood of winter crabs at 5° and 25° C. was not significantly different from the line of isotonicity. Over the salinity range, 6% to 125% sea water, blood concentrations for summer or

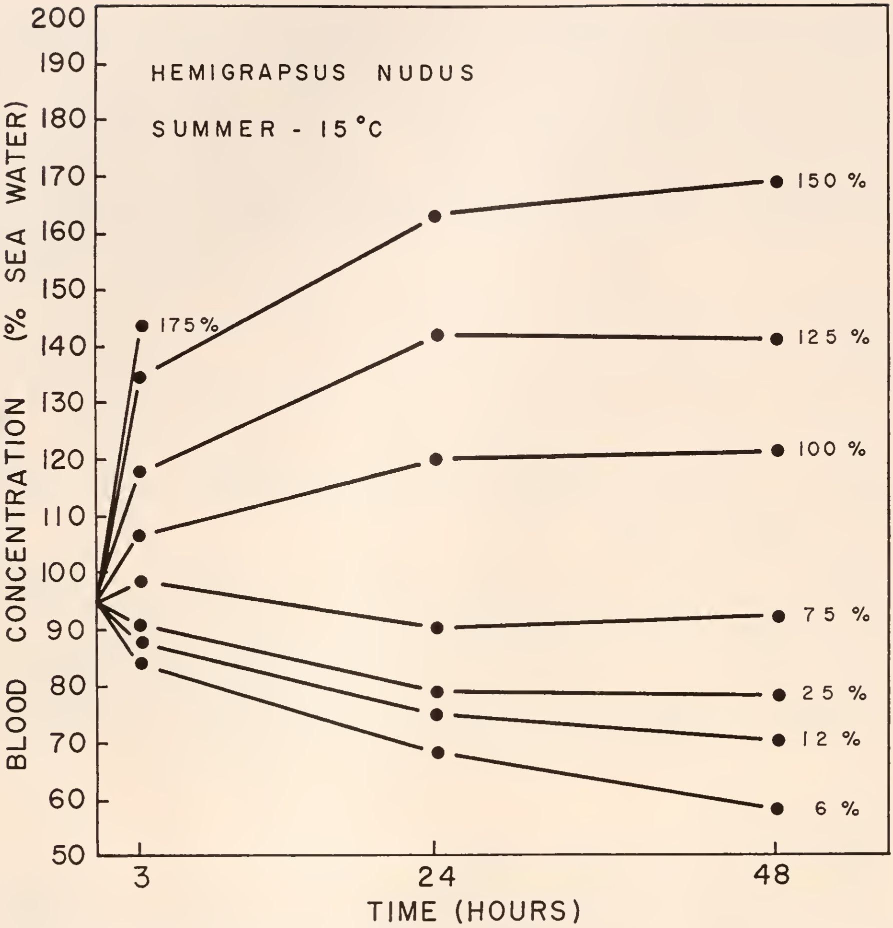

FIGURE 5. Osmotic regulation in summer *Hemigrapsus nudus* at 15° C., as a function of time in the experimental salinities. Each point represents the mean of the measurements of 10 to 15 animals for each time period. Sea water concentrations are indicated in per cent normal sea water for each of the respective curves.

winter crabs at the three temperatures were similar and not statistically significant, except for the 5° C. winter curve. This curve is noticeably higher throughout the intermediate range of salinities. At 125% sea water and above, the 5° C. curve loses its singular identity and the three temperature curves for winter animals are lower when compared with those of summer crabs. There was no real difference at each salinity when the blood concentration curves for summer crabs were compared at the three temperatures. The greatest difference in concentration effected by the maximum temperature difference (20° C.) was 14%, at a salinity of 12% sea water ($P = 0.01$), the usual being in the order of 5%. A greater blood concentration difference existed for winter animals over the range of salinity, 25% to 100% sea water. For instance, at 75% sea water, blood concentration differ-

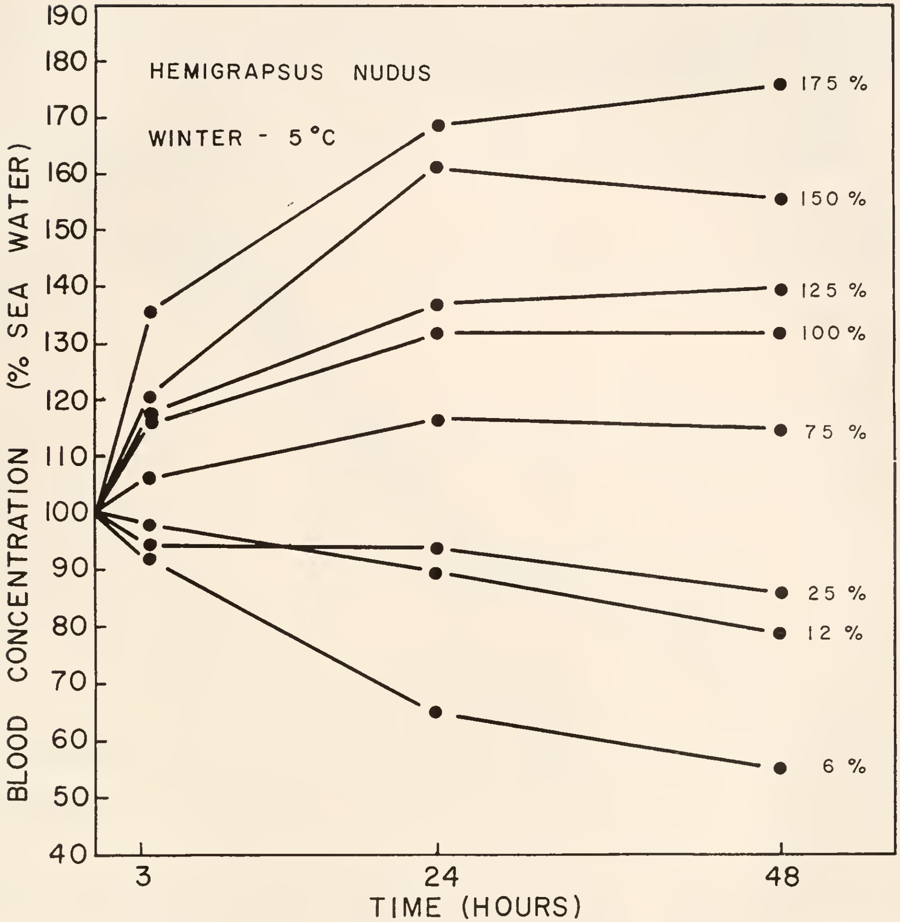

FIGURE 6. Osmotic regulation in winter *Hemigrapsus nudus,* at 5° C., as a function of time in the experimental salinities. Each point represents the mean of the measurements of 10 to 15 animals for each time period. Sea water concentrations are indicated in per cent normal sea water for each of the respective curves.

ence that resulted from the maximum temperature difference was 20% ($P = 0.01$). Minimum differences for winter animals ranged between 125% and 150% sea water. For summer animals this minimum difference occurred between 75% and 100% sea water. At the lower salinities, both summer and winter groups, the highest blood concentration generally was found at the lowest temperature, even though the temperature effect on blood concentration at a given salinity was relatively slight for summer animals.

Comparison of winter animals (Fig. 7) at their own temperature and salinity field conditions (5° C.; 75% sea water) with summer crabs (15° C.; 25% sea water) showed that winter crabs were more hypertonic (37% sea water difference; $P = 0.01$). Immediate testing of the blood of winter crabs, when removed from

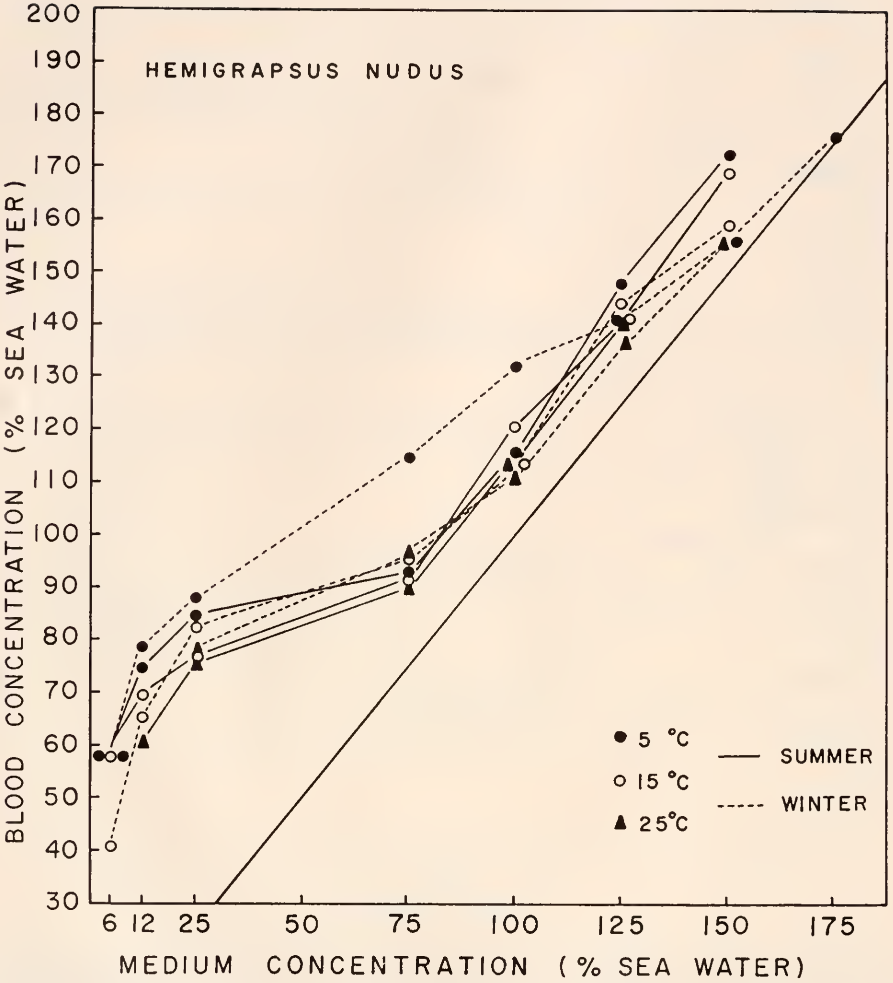

FIGURE 7. Relationship of the body fluid concentration of summer and winter *Hemigrapsus nudus* at 5°, 15° and 25° C., to the medium concentrations after exposure for 48 hours to the experimental salinities.

the intertidal zone, showed the blood concentration always to be higher when compared with summer crabs. And, at either season the blood concentration was higher than the intertidal salinity.

The concentration gradients between blood and external media for summer and winter *H. nudus* are presented in Figure 4. Winter crabs of this species maintained a greater gradient ($P = 0.01$) at sea water concentrations less than 125% except at 6%. At higher concentrations summer animals had the greater gradient ($P = 0.01$). As salinity increased winter crabs steadily decreased their gradient, and attained isotonicity at 175% sea water. The greatest gradient occurred at 12% sea water, for both summer and winter animals. At this low salinity, winter crabs maintained a 67% gradient and summer crabs a 57% gradient, and this difference is significant. Below 125% sea water, winter crabs are better regulators, and above this concentration, summer animals are better regulators. The differences at each salinity between summer and winter crabs are significant ($P = 0.01$), except at 6% and 125% sea water. Blood concentrations of summer and winter animals are significantly hypertonic ($P = 0.01$) to all external salinities (Fig. 7), except for winter animals at 5° and 25° C., 150% sea water, and 5° C., 175% sea water.

Mortality for summer *H. nudus* was somewhat similar to that reported for *H. oregonensis.* At 175% salinity deaths occurred at all temperatures, following three hours of exposure to the external medium. At 20° and 25° C. the same effect resulted at 150% sea water.

Interspecific comparison

Differences between the two species concern mainly seasonal changes and relative gradients maintained. Blood concentrations of summer *H. oregonensis* at the three temperatures, and salinities below 100% sea water, were similar, but significantly lower than those of winter animals (Fig. 3). Blood of summer *H. nudus* at the three temperatures, and salinities below 100% sea water, also was similar but not significantly lower than that of winter crabs, with the exception of the winter curve at 5° C. (Fig. 7). At salinities higher than 125% sea water, winter blood concentrations of both species were significantly lower and closer to isotonicity than corresponding summer ones.

Data presented in Figure 4 demonstrate clearly specific differences. Winter animals of both species maintained a greater gradient at salinities ranging from 6% to 100%–125% sea water. At higher external sea water concentrations summer animals of both species had the greater gradient. These differences between summer and winter animals of both species compared at each salinity are significant ($P = 0.01$) with the exception of *H. nudus* at 6% and 125% sea water and *H. oregonensis* at 100% sea water. Comparison of summer animals of both species showed that at salinities below 25% sea water *H. nudus* had a slightly greater gradient. From 25% to 75% sea water the two species had the same gradient. Between 75% and 125%, *H. nudus* was greater and at higher salinities, *H. oregonensis* sustained the greater gradient. At two salinities, 100% and 150% sea water, the differences between summer gradients were significantly different. The relationship between winter animals differed from that of summer ones. Between 6% and 50% sea water the gradient for *H. oregonensis* was greater. Over the range, 50% to approximately 150% sea water, the gradient for *H. nudus* was greater, and above 150% the *H. oregonensis* curve rose whereas that one for *H. nudus* decreased to isotonicity. Interspecific winter comparisons at each salinity are significant ($P = 0.01$) except at 150% sea water. Over the major portion of the salinity range, winter *H. nudus* is the better regulator, compared with summer *H. nudus* and summer and winter *H. oregonensis.*

Weight changes

Winter crabs of both species were placed at a series of salinities and at two temperatures (5° and 25° C.) for a 48-hour period, to determine whether an appreciable weight change occurred, particularly at the extremes of the salinity range (6% and 175% sea water). Percentage of body weight (dry and wet weight) as water was determined, and from these data it was shown that low and high salinities at either temperature had no effect on water gain or loss. Per cent of total body weight as water ranged from 59% to 65%, averaged 62%, and this range was not statistically significant. Animals were weighed within an average error of less than 0.5% of total body weight.

In all of the experimental procedures a weight range was selected to determine whether size affected time to equilibrium at various external sea water concentrations, and whether total osmotic pressure differed in various size groups. There was no evidence to suggest that size had any effect on the response of these two species of crabs to any of the salinity and temperature combinations, winter or summer.

Discussion

Comparative osmoregulatory abilities

The osmotic responses of these two species of crabs are different, and intraspecifically, the osmoregulatory ability changes seasonally. Over the entire salinity range blood concentrations of both species, summer and winter, and at the three experimental temperatures, are hypertonic to these experimental salinities and these differences, for the most part, are significant. As the gradient increases with decreasing salinity (below 100–125% sea water) osmotic regulation is accelerated (Figs. 3 and 7), and from 6% to 75% sea water both species show strong hyper-osmotic regulation.

The gradient between blood and external medium at various sea water concentrations shows a significant seasonal difference (Fig. 4). Gradients maintained by winter *Hemigrapsus oregonensis* and *H. nudus* are much greater, over the salinity range 6% to 100–125% sea water, when compared with those gradients for summer crabs. At higher salinities (100% to 150% sea water) summer crabs maintain a larger gradient. Differences between the two species become apparent when winter gradients are compared. At the lower (6% to 50% sea water) and higher (175% sea water) salinities, winter *H. oregonensis* have a significantly greater gradient. But over the major portion of the salinity range (50% to 150% sea water) winter *H. nudus* maintain the significantly greater gradient. Summer crabs are similar from 6% to 125% sea water. Only at the highest salinity (150% sea water) does *H. oregonensis* sustain a significantly greater gradient than *H. nudus.* The two summer curves are linear and parallel from 25% to 75% sea water; winter curves are nearly linear but not parallel from 12% to 125% sea water. The winter *H. nudus* curves approach linearity over most of the salinity range.

Jones (1941) found that both species of *Hemigrapsus* could regulate in dilute sea water, but even after 72 hours' immersion, he was unable to demonstrate regulation in concentrated sea water. Gross (1957a) confirmed the work of Jones (1941), but demonstrated further that *Hemigrapsus* can hypo-osmoregulate up to 33% (gradient between blood and external medium) for 20 hours in 150% sea water. He had shown that at 75% sea water both species maintain less than a 10% sea water gradient (calculated from Jones, 1941). It is assumed that at this salinity, isotonicity is most closely approached.

The present investigation supports the work of both Jones (1941) and Gross

(1957a), but demonstrates that regulation at higher and lower salinities is dependent upon the season, and blood concentrations are hypertonic to all experimental salinities. Summer *H. oregonensis* maintain a gradient up to 30% at 150% sea water, for longer than a 48-hour period. Summer *H. nudus* maintain one up to 20%. Winter *H. oregonensis* sustain a gradient up to 15% in 175% sea water, for the same period of time, but winter *H. nudus* are isotonic. At 75% sea water, the gradients of both species, summer and winter, particularly the latter, are much higher than those reported by Gross (1957a).

Gross (1960) recently has studied a transient population of *Hemigrapsus oregonensis* in southern California. A lagoon was isolated temporarily from the sea, and over a period of four months the salinity increased to 190% sea water. He has shown that in 168% sea water, this species is capable of hypo-osmotic regulation (gradient was 23%). At higher salinities the blood was nearly isotonic. These data differ from those presented here, in that this northern population is always hypertonic at all salinities. This condition of maintaining blood hypertonic to hypersaline media probably should not be referred to as regulation, in that it deviates from the normal understanding of homeostasis. The fact that per cent body weight as water is not affected by various sea water concentrations suggests that at high salinities, salts are being absorbed from the external medium and this maintains hypertonic blood. This hypertonicity, however, may result from a gradual breakdown of the regulatory mechanism. Gross (unpublished) acclimated a group of *Hemigrapsus oregonensis*, over a period of two months, from a field salinity of 163% sea water to 100% sea water. Following this, these animals were immersed directly into 150% sea water for 72 hours. These animals could not regulate at this salinity. He has suggested that long term acclimation has permitted hypo-osmotic regulation in this species.

Effect of temperature

There is little evidence available regarding the effect of temperature on osmotic regulation. Jones (1941) has shown that a higher temperature causes a slightly higher osmotic pressure. He states (p. 85) that "the magnitude of the change is not great as compared with the individual variations at a given temperature." For instance, over a 20° C. range, the increase in the hyper-osmotic regulation curve for *Hemigrapsus oregonensis* and *H. nudus* is approximately 5% to 7% sea water. Bateman (1933) showed in *Carcinus maenas* an increase in osmotic pressure with an increase in temperature. The increase was slight; with a temperature range of 14° C. the increase was approximately 1% sea water. Widmann (1935) found in *Eriocheir sinensis* an increase in osmotic pressure with a decrease in temperature. But again the increase was small. More recently, Williams (1960) has studied the effect of temperature on osmotic regulation in two shrimps, *Penaeus aztecus* and *P. duorarum*. He reported that as temperature was lowered from 28° to 18° C. and 18° to 8° C., blood concentration approached isotonicity at all experimental salinities (10‰ to 30.5‰). He stated further that at low temperatures (8° C.) the regulatory ability was impaired. It is difficult to compare the response at different temperatures because the time at each temperature was not the same. Gross (unpublished), however, has demonstrated in *Dendrostomum* that blood Ca and Mg are significantly higher at lower temperatures (3.5° C.) when compared with higher ones (13.5° C.).

From the foregoing data, it would appear that a temperature effect is yet to be clearly documented with the exception of that reported by Gross (unpublished). The seasonal effect on osmotic regulation in *Hemigrapsus* has been demonstrated in this paper (Figs. 3, 4 and 7). These differences are the result of temperature. Within either species, summer animals at lower salinities are consistently lower in blood concentration than winter animals, and at higher salinities, summer animals are consistently higher. Over the salinity range (25% to 75% sea water) encountered seasonally by these populations, the difference between the summer and winter gradient of *H. oregonensis* at 25% sea water is 21%; at 75% sea water, the difference is 10%. The same comparison for *H. nudus,* at 25% sea water, is 8%; at 75% sea water, 23%. The differences in these gradients are significant ($P = 0.01$). The two summer curves from 25% to 75% sea water are identical, and can be used as a baseline (Fig. 4). As salinity increases, low temperature (winter) causes a sharp decrease in the gradient (curve steepens) for *H. oregonensis,* and conversely, low temperature causes a more gradual decrease for *H. nudus.*

Comparison of Figures 3 and 7 shows, in both cases, a difference in the 5° C. winter curve over the salinity range, 25% to 75–100% sea water, depending upon the species, when this curve is compared with all others. Any significance attached to this difference can be determined only after specific ion analyses, research on which is proceeding at present.

Metabolic work in increased osmotic stresses

Recently, studies on whole animal respiration of *Hemigrapsus oregonensis* and *H. nudus,* as influenced by various temperature and salinity combinations, have shown that for a given salinity, animals acclimated to low temperature (5° C.) have a higher rate of metabolism. Further, crabs acclimated to a given temperature have a higher rate at lower salinities (Dehnel, 1960). Weight-specific oxygen consumption is highest at the low acclimation temperature, low acclimation salinity combination. Rates of oxygen consumption remain high as temperature increases in the low salinity for *H. oregonensis,* but high salinity results in a higher rate for *H. nudus.* The greatest gradient maintained by either species, summer or winter, is at a low salinity, 12% sea water (Fig. 4). In order to maintain a gradient of this magnitude (hyper-osmoregulate) some metabolic work must result. This does not define, however, the percentage relative to total metabolism of the whole animal.

Potts (1954) has discussed recently the dynamic aspects of osmoregulation and has pointed out that urine hypotonic to the blood in brackish-water animals has only a slight effect on osmotic work. But, if in fresh water, urine is greatly concentrated relative to the medium, and still hypotonic to the blood, osmotic work is reduced significantly. Both species of *Hemigrapsus* in this geographic area are found only in brackish or estuarine water, and in the summer estuarine conditions become more pronounced (salinity, 25% to 35% sea water). These seasonal changes present ecological conditions intermediate to brackish and fresh water as discussed by Potts (1954). Over the experimental salinity range, blood of winter animals of both species is always hypertonic to the urine. The blood of summer crabs of both species is isotonic at lower salinities and slightly hypertonic at higher salinities.

It is possible to compare respiration rates and osmoregulatory data at various temperature and salinity combinations in order to determine whether one can accord increased metabolic activity to responses to salinity where large gradients are maintained. Whole-animal respiratory rates for summer animals of both species, at a series of acclimation temperatures and salinities, are available to relate these values to the present data, specifically the hyper-osmotic regulation portion of the salinity curves (Dehnel, 1960). If the osmotic gradients of summer animals, determined at 5° (Figs. 3 and 7) and 20° C. (data not presented) and 25% and 75% sea water, are compared with weight-specific oxygen consumption values for summer animals acclimated to the same temperature and salinity conditions (Dehnel, 1960), the following relations can be shown. For *H. oregonensis* at 5° and 20° C. there is 129% and 157% increase in gradient, respectively, as salinity decreases from 75% to 25% sea water. Metabolic rate accordingly increases as salinity decreases and at either temperature, by essentially a constant value, 28%. The gradient increase for *H. nudus,* compared as above, is 233% at 5° C. and 146% at 20° C. Oxygen consumption at the lower temperature shows a 31% increase, but at 20° C., even though the gradient has more than doubled, respiratory rate has decreased 20%. In all examples the gradient increases as salinity decreases, and except for *H. nudus* at 20° C., there is a parallel increase in oxygen consumption, and the magnitude of increase is approximately the same.

An important reference point is the comparison of blood and urine at various experimental salinities. One aspect of the work currently in progress (to be published elsewhere) concerns changes in total osmotic pressure of the urine of *Hemigrapsus* under identical conditions to those described for blood. When the gradient between the blood and urine is compared seasonally (plotted as in Figure 4), winter *H. oregonensis* and *H. nudus* have blood hypertonic to urine over the entire salinity range. There exists at least a 10% absolute difference, and in some cases the difference is as high as 35%. Blood of summer animals of both species is isotonic with the urine at salinities ranging from 6% to 100% sea water, and is slightly hypertonic at sea water concentrations above 100% sea water. If the gradient between urine and the external medium is compared, winter *H. oregonensis* maintain a greater gradient than summer crabs, at lower salinities, and at higher salinities urine of winter *H. oregonensis* is hypotonic. Above 100% sea water urine of summer crabs is hypertonic to the external medium. The gradient for summer and winter *H. nudus* from 25% to 75% sea water is similar. Above this salinity, summer and winter *H. nudus* follow the same trend as described for summer and winter *H. oregonensis,* although the latter are somewhat higher in urine concentration at each of the respective higher salinities. The general trend for blood and urine is the same with the exception that urine of winter crabs becomes hypotonic at 100% sea water and above, whereas blood concentration always remains hypertonic.

Several considerations arise from comparison of these data. The fact that oxygen consumption increase is approximately the same, whereas the percentage increase in gradient at the low salinity is different, suggests that increased osmotic work cannot be detected by measuring whole-animal oxygen consumption, at least under these conditions. The exception, namely *H. nudus* at 20° C., documents further the above. Summer gradients of either species at both temperatures compared at 25% or 75% sea water are similar, and interspecifically, these species in the summer hyper-osmoregulate to a comparable degree. This is seen in Figure 4 at 15° C. The reasons for the decrease in oxygen consumption at 25% sea water, 20° C. for *H. nudus* cannot be explained.

Mechanism of osmoregulation

Prosser, Green and Chow (1955) have presented evidence for an osmotic regulatory role of the antennary gland in *Pachygrapsus.* The kidney functions probably only for hyper-osmotic regulation and even more selectively for the regulation of Ca and Mg ions. Green, Harsch, Barr and Prosser (1959) have demonstrated a similar role, especially for Mg ions, for the antennary gland of *Uca.* Gross (1960) has demonstrated high Mg urine concentrations for *H. oregonensis.* The selective role of the kidney for ionic regulation is being determined for these northern populations of the two species of *Hemigrapsus.* These data suggest that the kidney functions for the regulation of Ca and Mg ions, and probably in both low and high salinities.

The role of the gill for osmotic regulation is well documented. Gross (1957a) has demonstrated a flux of salts and water in the gill chamber of *Pachygrapsus.* Green, Harsch, Barr and Prosser (1959) have shown that water and ions enter via the gills and stomach of *Uca* and the sites of regulation are the gills and the antennary gland (see above). There are no data available at present to suggest the role of the gill in osmotic regulation in *Hemigrapsus.* In this laboratory, rate of respiration of gill tissue has been determined for summer and winter crabs of both species, over a range of temperatures and salinities. These data show that gill tissue respiration of crabs measured at a series of acute temperatures, and acclimated to 5°, 12.5° and 20° C. and 35% to 125% sea water, decreased generally as salinity increased, and the decrease was greatest in summer animals acclimated to 5° C.

There were no detectable weight changes in winter crabs in the experiments conducted at two temperatures (5° and 25° C.) and at eight salinities (6% to 175% sea water). Per cent of the total body weight as water was approximately 62%. These data indicate that there was no net gain or loss of water, but if water does change blood concentration, its effect is negligible. This is an agreement with Hukuda (1932) and Gross (1957a). If, then, water fluxes do not occur, it is reasonable to assume that body fluid concentration changes result from salt movement. At the lower salinities (6% to 75% sea water) salt concentration of the blood was maintained actively against a gradient in both species, winter and summer (Figs. 3 and 7). Between 75% and 100% sea water, the blood continued to maintain a gradient, although this gradient decreased as the salinity increased. Blood of animals removed directly from field conditions was essentially 100% sea water, even though field salinities, summer and winter, are approximately 25% and 75% sea water, respectively. At experimental salinities above 100% sea water, blood concentrations remained hypertonic and were maintained against a gradient.

The present data permit no further interpretation of the above results. Work currently in progress on major ion changes in blood and urine for the two species and at the various temperature and salinity combinations will demonstrate the selective role of the kidney, temperature effect on ion exchange, and the hypertonic condition of the blood at higher salinities.

Intertidal distribution

It has been suggested that seasonal and laboratory responses of *Hemigrapsus nudus* to combinations of temperature and salinity, as measured by whole animal oxygen consumption and temperature tolerance, might explain the establishment of this species in this geographic area (Dehnel, 1960). Winter *H. nudus* maintain a greater gradient when compared with *H. oregonensis,* over an approximate salinity range, 50% to 150% sea water. At lower and higher salinities, *H. oregonensis* has a higher gradient. Gradients for summer populations of both species were approximately the same (Fig. 4). Based on these gradients *H. nudus* is the better regulator.

Gross (1957b) has demonstrated that *Pachygrapsus crassipes* prefers 100% sea water to other concentrations, ranging from 50% to 150% sea water. This preference was not altered by desiccation or acclimation to low salinity, but could be changed with high salinity acclimation. He suggests that this preference may serve as a mechanism for limiting *Pachygrapsus* to the intertidal zone.

Studies on salinity preference have shown that winter *H. nudus* has no preference for the three salinities tested, 25%, 75% and 125% sea water, whereas *H. oregonensis* showed a distinct preference for 75% sea water, the field salinity for winter animals.

Gross (1957a) has found a direct correlation between exoskeleton permeability and osmotic regulation in a series of crabs, habitats of which range from marine to terrestrial. In this series of crabs, the exoskeleton of *H. nudus* was shown to be less permeable than that of *H. oregonensis.*

Winter breeding of both species of *Hemigrapsus* occurs at this latitude. Zoea and megalops larvae of *H. nudus* from the open coast could have introduced this species into this geographic area, and winter temperature and salinity conditions would not restrict the establishment of a population in the intertidal region. The better regulatory ability of *H. nudus,* lack of salinity preference for winter animals, in conjunction with the less permeable exoskeleton, would allow this species to locate higher in the intertidal regions, where there is a greater osmotic stress, but reduced competition with the population of *H. oregonensis* that is established in the lower tidal areas. Habitat requirements of the two species differ slightly, but are not sufficient to restrict considerable overlap of the two populations.

SUMMARY

1. Total osmotic pressure measurements of blood were made on two species of intertidal crabs, *Hemigrapsus oregonensis* and *H. nudus,* over a salinity range, 6% to 175% sea water, a temperature range, 5° to 25° C., and at two seasons, summer and winter.

2. Major changes in blood concentration occurred at 48 hours. Both species at either season were hypertonic to all experimental salinities. Below 100% sea

water, osmotic regulation was accelerated with decreasing salinity, and in hypotonic media both species showed strong hyper-osmotic regulation.

3. The osmoregulatory abilities of these two species changed seasonally, and these responses resulted from the effect of temperature. Winter crabs maintained greater gradients at sea water concentrations below 100–125% sea water, and were better regulators in hypotonic media, whereas summer crabs were better regulators in hypertonic media, and consequently maintained the greater gradient. Interspecifically, winter *H. nudus* maintained the greater gradient over the major portion of the salinity range. Summer crabs of both species were similar from 6% to 125% sea water.

4. Blood concentrations of *H. oregonensis,* when measured at a series of temperatures and at each salinity, showed a general trend, particularly for winter animals. As external sea water concentration decreased (from 75% to 12% sea water) blood concentrations increased significantly with decreasing temperature. Blood concentrations of summer animals measured at the three temperatures and each salinity showed no real difference, but at the lower salinities, blood concentrations of summer animals were significantly lower than those of winter crabs. The same general trend was shown for *H. nudus.* Temperature had no effect on blood concentrations of summer animals, but the low temperature (5° C.) had a highly significant effect on the blood concentrations of winter animals.

5. No detectable weight changes resulted when animals were subjected to the extreme experimental sea water concentrations. Further, there was no evidence to suggest that size had any effect on osmotic response.

6. Respiratory and osmoregulatory data for both species have been compared at the same temperatures and salinities to determine whether increased osmotic work can be resolved by measuring oxygen consumption. Comparison of these data does not permit such a conclusion.

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IONIC BALANCE IN BLOOD AND COELOMIC FLUID OF EARTHWORMS[1]

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The osmotic relations in earthworms have been studied by several investigators and have been reviewed by Ramsay (1949a). It is well established that earthworms behave osmotically like fresh-water animals. Water enters the body through the skin and is eliminated by the nephridia with the production of a hypotonic urine (Bahl, 1947; Ramsay, 1949a, 1949b).

Only sporadic reports are available on the ionic balance in earthworms. Bahl (1947) reported the ionic concentrations of the body fluids of *Pheretima posthuma,* indicating that the sodium concentration of the coelomic fluid was twice as concentrated as that of the blood. He also showed that the chloride concentration of the blood was lower than that of the coelomic fluid, the concentration being only about a third of that of the sodium concentration. Ramsay (1949a) reported the concentration of chloride in the blood and coelomic fluid of *Lumbricus terrestris.* He showed that the chloride concentration of the blood was slightly lower than that of the coelomic fluid. Prosser and Brown (1961) cite values for sodium and potassium in the body fluids of *Lumbricus,* showing a higher concentration of sodium and a lower concentration of potassium in blood as compared to coelomic fluid.

This paper is concerned with the balance of sodium, potassium, calcium and chloride in the blood and coelomic fluid of earthworms.

Materials and Methods

Three species of earthworms were used in this study. *Lumbricus terrestris* was obtained from the Carolina Biological Supply Co. *Eisenia foetida* was collected from manure compost on campus, while *Helodrilus caliginosus* was obtained locally from bait dealers. All animals were kept either in moist soil or aerated tap water in large aquaria for a week before being used in any of the experiments. The animals were kept at 16° C. and all experiments were carried out at this temperature. Only large adult worms were used.

Blood and coelomic fluid were collected in the following manner. A drawn capillary was inserted directly into the coelom through the body wall and the coelomic fluid was readily collected. The earthworm was then anesthetized individually with 15% ethanol. It was found that anesthetizing the animal does not affect the ionic concentration of the blood. The body wall was then cut dorsally and pinned out on a waxed pan to expose the dorsal blood vessel and aortic arches. The coelomic fluid was blotted away and a drawn capillary was inserted into either the dorsal blood vessel or aortic arch. By this method, it was possible to collect

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a sample of blood sufficient for the analyses of the cations and chloride. Blood was collected only from *L. terrestris* and *H. caliginosus.* Because of the size of *E. foetida,* blood was not collected from this species.

Samples of blood and coelomic fluid were centrifuged and the supernatant fluid was analyzed for sodium, potassium and calcium with a Coleman flame photometer. Chloride was determined with an Aminco-Cotlove chloride titrator.

Body fluids of animals kept in moist soil, tap water and 0.1 *M* NaCl solution were analyzed for ionic concentrations in *L. terrestris* and *E. foetida. H. caliginosus* from moist soil was studied for comparison.

RESULTS

The results are presented in Table I. The concentrations of sodium, potassium and calcium are significantly higher in the blood than in the coelomic fluid in all groups studied. There is no difference in the concentrations of chloride in the body fluids. The chloride concentration is about 50% of the sodium concentration. With exposure to 0.1 *M* NaCl solution, the concentration of sodium in both the blood and coelomic fluid is increased and maintained higher than the concentration in the medium. There is almost no difference in the ionic concentrations in the three species studied under the same conditions.

DISCUSSION

The data presented in this study demonstrate that there is regulation of sodium, potassium and calcium concentrations between the blood and coelomic fluid of earthworms. The concentrations of these ions are always greater in the blood than in the coelomic fluid. This relationship has been pointed out most recently by Prosser and Brown (1961), who have cited a higher value for sodium in the blood than in the coelomic fluid of *Lumbricus.* However, a higher value for potassium is cited in the coelomic fluid. No experimental details were presented. Bahl (1947) reported a concentration of sodium in the coelomic fluid twice that in the blood of *Pheretima posthuma.* His value for sodium in coelomic fluid (80 meq./l.) is comparable to the values for the three species reported in this paper. He also reported a higher concentration of calcium and chloride in the coelomic fluid but lower values of potassium as compared to blood. There is no difference in the concentration of chloride between the body fluids studied in *H. caliginosus* and *L. terrestris.* Ramsay (1949a) compared the chloride concentrations in two animals of *L. terrestris* and reported that the blood chloride was slightly lower than in the coelomic fluid. His values (43 and 46 meq./l. in blood and coelomic fluid, respectively) are comparable to the values obtained for this species in this study.

The sodium concentration of the body fluids is maintained higher than that of the environment when the animal is placed in 0.1 *M* NaCl, a concentration greater than that of the body fluids of worms in soil or tap water. Ramsay (1949a) has shown that although the osmotic pressure of the coelomic fluid, as measured by the freezing point method, remains higher than the concentration in the medium as high as 0.24 *M* NaCl, the chloride concentration becomes hypotonic to the medium at an external concentration of 0.055 *M* NaCl. The concentration of sodium is

TABLE I

Ionic concentrations in blood and coelomic fluid of earthworms

	Ionic conc. (meq./l.)						
	No. animals	Coelomic fluid		No. animals	Blood		p
		Mean	SD		Mean	SD	
Lumbricus terrestris							
Moist soil							
Na	7	75.6	4.5	7	85.7	6.6	.01
K	7	4.0	1.5	7	5.5	1.0	.05
Ca	7	5.9	1.7	7	16.7	3.1	.001
Cl	7	42.8	2.6	10	39.0	8.3	.20
Tap water							
Na	24	78.1	2.9	20	83.6	3.5	.001
K	24	2.7	0.7	20	5.3	1.3	.001
Ca	23	4.6	1.1	8	12.2	1.4	.001
Cl	16	48.5	4.8	14	47.2	4.7	.70
0.1 *M* NaCl							
Na	10	112.7	3.6	8	122.1	10.1	.02
K	10	3.3	0.5	8	7.0	0.8	.001
Ca	10	8.3	1.3	5	14.8	1.1	.001
Cl	10	74.4	6.2	8	77.7	11.2	.70
Helodrilus caliginosus							
Moist soil							
Na	14	78.6	5.1	10	88.5	7.7	.001
K	14	5.8	1.6	10	7.9	0.8	.001
Ca	14	7.3	1.6	10	26.7	6.5	.001
Cl	15	35.6	3.0	8	35.9	2.7	.80
Eisenia foetida							
Moist soil							
Na	20	79.7	4.8				
K	20	4.0	0.9				
Ca	20	8.0	2.1				
Cl	20	42.0	6.7				
Tap water							
Na	16	70.0	7.3				
K	16	3.5	1.1				
Ca	16	5.6	0.8				
Cl	10	42.0	6.7				
0.1 *M* NaCl							
Na	12	116.5	3.9				
K	12	3.6	0.8				
Ca	12	6.8	2.6				
Cl	11	88.4	3.6				

approximately twice that of chloride in the body fluids. This would mean then that the earthworm can maintain hypertonicity with respect to sodium to 0.11 *M* NaCl in the medium. It is shown in Table I that sodium in the body fluids of earthworms remains hypertonic to the medium at an external concentration of 0.1 *M* NaCl.

It is interesting to note that the chloride of both the blood and coelomic fluid is about 50% of the concentration of sodium. This phenomenon has been reported by Bahl (1947) for *Pheretima posthuma.* Maluf (1940) showed that the chloride concentration of a mixture of blood and coelomic fluid (46 meq./l.), considered as NaCl, can account for only 29% of the total osmotic pressure of this mixture. Ramsay (1949b) reported that NaCl, based on chloride concentrations, can account for about 50% of the total osmotic pressure of the coelomic fluid, assuming that sodium and chloride form a large proportion of inorganic substances present. The sodium concentration was not determined. Both authors suggest that organic substances are responsible for the remaining fraction of the total osmotic pressure. The osmotic pressure of tap water animals is given as 90 meq. NaCl/1. (Ramsay 1949a). It should be noted that the total concentration of the three cations studied in the coelomic fluid of tap water animals is 85 meq. (Na, K, Ca)/1. It seems probable that the inorganic ions do contribute to most of the total osmotic pressure in earthworm body fluids. Anions other than chloride then must be responsible for total electrolyte balance.

SUMMARY

1. The concentrations of sodium, potassium, calcium and chloride in the blood and coelomic fluid were determined in *Lumbricus terrestris* kept in moist soil, tap water and 0.1 *M* NaCl solution, and in *Helodrilus caliginosus* kept in moist soil. Coelomic fluid only was analyzed in *Eisenia foetida.*

2. The concentrations of cations are higher in the blood than in the coelomic fluid in all groups studied. There is no difference in the concentration of chloride between the blood and coelomic fluid.

3. Ionic concentrations are similar in the three species studied under similar conditions.

4. The concentration of sodium in both blood and coelomic fluid is maintained greater than the medium in 0.1 *M* NaCl solution.

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LOCALIZATION AND PROPERTIES OF PHOSPHOPROTEIN PHOSPHATASE IN THE FROG EGG AND EMBRYO[1]

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Phosphoprotein phosphatase (PPPase) has been suggested to be important in the mechanism of yolk utilization in the frog embryo (Harris, 1946; Barth and Barth, 1954; Flickinger, 1956, 1957). Knowledge of the mechanism of regulation of the enzymatic activity during development must follow from a thorough understanding of the properties, location and changes in activity which occur during morphogenesis. In view of the unsettled nature of the literature concerning these areas an investigation has been made into various phases of these problems.

The use of different fractionation and incubation procedures upon ovarian eggs has resulted in PPPase activity being found in the yolk (Harris, 1946; Nass, 1956), the small yolk (Panijel, 1950), the pigment-containing fraction (Barth and Barth, 1954) and in water-soluble dephosphorylated vitellin (Flickinger, 1956). All of these investigators have reported enzymatic activity in several of these fractions of ovarian homogenates. There have been reports on calcium activation of the enzyme (Flickinger, 1956) and on calcium inhibition (Nass, 1956). The phosphate release from vitellin has also been suggested to be non-enzymatic (Flickinger, 1956).

Mezger-Freed (1953) studied the changes in the endogenous phosphate released from embryo homogenates during development and found peak activities just before and during gastrulation and a steady decline after neurulation. Flickinger (1956) reported a stage-wise increase in PPPase activity from eggs to hatched larvae. Barth and Barth (1951) found a low enzymatic activity of KCl extracts until gastrulation when a slight increase was observed. The activity reached its peak at hatching and declined thereafter.

Yolk has also been suggested to play a primary role in morphogenesis. It has been shown that the yolk is important in determining the polarity of the egg (Ancel and Vintemberger, 1948), and Dalcq and Pasteels (1937) have hypothesized that the product of the interaction of a cortical and vitellin gradient determines the fate of the various regions of the embryo. Most microscopical investigations of yolk utilization indicate that the yolk platelets are solubilized most rapidly at the time of hatching, although evidence for an earlier utilization of yolk has also been reported (Voss, 1934; Bragg, 1939).

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There is excellent agreement that the yolk is first used in the most actively differentiating regions, especially the chorda-mesoderm (Bragg, 1939; Flickinger, 1949, 1954). Flickinger (1949) has correlated the disappearance of yolk with the initiation of pigmentation of neural crest pro-pigment cells, as well as with increased oxygen consumption that follows yolk utilization. Electron microscopy of amphibian eggs and embryos shows that an intimate relationship exists between yolk and mitochondria, and Lanzavecchia and Le Coultre (1958) have suggested that a direct conversion of yolk to mitochondria takes place. Karasaki (1959) has shown that the mitochondria increase in number and complexity from late gastrulation onward, at the time when yolk utilization and oxygen consumption increase.

The major part of this investigation is concerned with clarifying the problem of the localization of PPPase in the ovarian egg and characterizing its properties after freeing the enzyme from its endogenous substrate. The changes in the enzyme activity during development have been investigated and correlated with the localization studies and with the known morphogenetic movements occurring at various stages of development.

MATERIALS AND METHODS

A. *Preparation of fractions*

1. *Yolk fractions.* Ovaries of *Rana pipiens* were excised, washed and homogenized in cold 0.05 M Tris-maleate buffer, pH 6.8, which in some cases contained 0.5 M NaCl. The ovary and oocytes were disrupted in one or two strokes of the pestle. Excessive homogenization results in pigment adsorption upon the yolk platelets, as previously described (Panijel, 1950; Nass, 1956). All procedures prior to incubation were performed at temperatures below 4° C. Yolk platelets were isolated according to the method described by Essner (1954), with minor modifications in the centrifugation schedules (Nass, 1961). All preparations were routinely examined microscopically for uniformity and absence of contaminants.

Small yolk platelets were best prepared by removing the bulk of the large yolk at 120 g and then centrifuging the supernatant fraction at 700 g. The sediment was re-suspended, centrifuged to remove traces of the larger yolk components, and the small yolk was re-sedimented. This procedure was repeated at least five times. In many preparations this small yolk fraction remained gray from pigment adsorption even after ten washings with buffer. Microscopically, the fraction was found to contain few large yolk platelets.

In some instances a combined pigment-small yolk fraction was prepared by centrifugation at 4000 g for 10 minutes after previous removal of the large yolk fraction.

A few preparations of large and small yolk by the method described by Panijel (1950) were attempted. In most instances the sucrose medium caused aggregation of the yolk and was not as satisfactory in the separation of the platelets as the method described above. The procedure of centrifuging all the components down at a higher centrifugal force and then re-suspending them also led to excessive, irreversible pigmentation of the yolk fractions.

2. *YP_A and ghosts.* Clean yolk platelets are almost completely and immediately soluble in solutions of ionic strength greater than 0.4 M. The small amount of insoluble material (ghosts) was centrifuged down and the soluble yolk re-precipi-

tated by lowering the salt concentration to 0.10 *M* or less by dilution or dialysis against distilled water or dilute buffer, pH 6.8. This material is referred to hereafter as "YP_A" and is equivalent to the YP described by Gross (1954).

3. *YP_B.* When ovaries were homogenized in buffer containing 0.5 *M* NaCl, the first centrifugation was carried out at 10,000 *g* for 15 minutes in order to remove most of the particulate matter while leaving the soluble yolk proteins in the supernatant fluid. The large quantity of lipid-containing material separating centripetally was skimmed off and discarded; the remainder of the supernatant fluid was dialyzed against distilled water or dilute buffer for 24 hours in order to precipitate the yolk proteins. This material is called "YP_B" and is similar to the preparations described by Barth and Barth (1954).

4. *Pigmented fraction and extract.* The gummy, black residue after extracting and centrifuging the ovaries was re-homogenized in buffer containing 0.5 *M* NaCl and centrifuged at 4000 *g* for 10 minutes. The procedure was repeated twice in order to remove most traces of adsorbed yolk proteins. This material was sometimes used as the enzyme source, but part of it could be dissolved by extraction with pH 4.9 acetate buffer as described by Barth and Barth (1954). The residue was extracted three times in small volumes of buffer with centrifugation and suspending of the material between each extraction.

5. *Embryonic material.* Eggs and embryos of the desired stage of development (Shumway, 1940) were washed and homogenized in three volumes of 0.1 *M* Tris-maleate buffer, pH 7.2, containing 0.5 *M* KCl. The homogenate was centrifuged and the fractions were prepared as described above.

B. *Storage of fractions*

Yolk platelet organization and properties are disrupted by freezing or acetone powder preparation, and whole ovaries are no longer extractable after freezing. Yolk held at 4° C. releases some phosphate during storage (Panijel, 1950; Nass, 1956). It was found that whole ovaries, intact platelets, yolk platelets soluble at high ionic strengths, and insoluble residues maintained their physical and enzymatic properties for as long as one year when stored in 50% glycerol at −10° C. Material that was prepared for storage was made up in double concentrations of Tris-maleate buffer, pH 6.8, and/or NaCl and then diluted in half with glycerol. For use, the glycerinated material was either diluted or dialyzed against distilled water, centrifuged, washed and re-diluted with the desired buffer.

C. *Substrates*

Phosvitin (Nutritional Biochemical Co.) was used routinely as the substrate because of its favorable solubility properties over the pH range employed. Yolk platelets or solubilized yolk could be used as a substrate after heating at 65–70° C. for three minutes. Most of the enzymatic activity of the yolk was destroyed by this treatment, while the yolk retained its characteristic solubility properties at weak and strong salt concentrations. Heating at higher temperatures irreversibly denatured the proteins.

D. *Analytical procedures*

All fractions were incubated with and without added substrate in both 0.1 *M* and 0.5 *M* NaCl. Controlled variables included enzyme concentration, substrate concentration, ionic strength, pH, temperature, and time of incubation. Most incubations were performed at pH 4.9 for 15 minutes at 28° C. The reaction was stopped by adding 10% trichloroacetic acid (TCA) to yield a final concentration of 7.5%. The samples were centrifuged and an aliquot removed and analyzed for inorganic phosphate by the Fiske-Subbarow procedure, as modified by Leloir and Cardini (1957). The addition of molybdate to a TCA extract of frog eggs or yolk resulted in a clouding of the originally clear extract, as described by Harris (1946) and Mezger-Freed (1953). This necessitated an additional centrifugation before the reducing solution was added.

The total phosphorus method employed was described by Leloir and Cardini (1957), but superoxal (30% hydrogen peroxide) was substituted for nitric acid.

The fractionation of phosphorus-containing compounds was performed by the Schneider (1945) technique.

Soluble protein was sometimes determined by the biuret procedure described by Gornall *et al.* (1949) after standardization of representative samples by the Kjeldahl procedure. Most nitrogen analyses were performed by the micro-Kjeldahl method.

Experiments and Results

The wide variations in the localization of PPPase were studied by comparing the techniques of other investigators (Barth and Barth, 1954; Flickinger, 1956; Panijel, 1950) with our own (Nass, 1956). The experiments reported here are consistent with the view that endogenous PPPase activity of yolk is due to adsorption of pigmented material and that the stability of solubilized yolk is dependent upon the previous steps in the isolation procedure. The described kinetics, pH optima, temperature characteristics, substrate specificity and inhibition studies on fractions freed of endogeneous activity are positive indicators of a true enzymatic hydrolysis of phosphoprotein phosphate.

A. *Observations on yolk fractions prepared by different methods*

The data summarized in Table I show that salt extraction of whole homogenates resulted in a dephosphorylation of the yolk (YP_B) phosphoprotein. A concomitant alteration in the solubility properties of these preparations was also noted. The loss of phosphate from phosphoprotein renders the protein water-soluble (Flickinger, 1956; Nass, 1956). Solubilization of clean yolk (YP_A) followed by centrifugation and re-precipitation by dilution with water showed little loss of phosphoprotein phosphorus, and complete precipitation of the extracted yolk was obtained. Further solubilization and re-precipitation left trace quantities of high-salt-insoluble material in YP_A but large quantities of this material in YP_B. Other evidence of the instability of YP_B phosphoprotein includes the solubility of this material at both acid and alkaline pH as compared with the acid precipitability of YP_A. Upon incubation at pH 4.9, 28° C., YP_A lost phosphate and became water-soluble.

TABLE I

Phosphorus fractions of yolk preparations

	Total P	Acid-soluble P tot. = P_i + true acid-sol.			Lipid P	RNA P	Phosphoprotein P	Phosphoprotein P + P_i
mg. P/mg. N								
WY*	0.14	0.021	0.017	0.005	0.039	0.014	0.065	0.082
YP_A	0.14	0.026	0.021	0.005	0.045	0.016	0.062	0.083
YP_B	0.12	0.047	0.042	0.005	0.032	0.022	0.015	0.057
% total P								
WY*	100	15	12	3	28	10	46	58
YP_A	100	19	15	4	32	11	44	59
YP_B	100	38	34	4	26	18	12	46

* Whole yolk platelets.

The instability of YP_B phosphoprotein was observed in well buffered (pH 6.8) media and at temperatures not exceeding 4° C., at which PPPase activity is negligible. Other non-enzymatic dephosphorylations have been noted in stored whole yolk (Nass, 1961; Harris, 1946) and in heated yolk where the enzymatic activity had been destroyed. The supernatant fraction of all homogenates contained large quantities of P_i (20% of the total phosphate of the egg), which perhaps were released during the original homogenization procedure.

YP_B preparations of embryonic yolk proteins were found to be much more stable than ovarian YP_B preparations. The endogenous activity of these fractions was much lower, and they were noticeably freer of pigment contaminants than ovarian YP_B.

B. *Localization studies of PPPase in ovarian fractions*

The following experiments provide evidence that PPPase is localized in the pigment fraction and that the endogenous PPPase activity of yolk results from adsorption of pigmented material upon the yolk platelet surface.

(1) The endogenous enzyme activity of similarly prepared yolk fractions was variable when the protein concentrations were equated (Nass, 1956, 1961).

(2) Figure 1 shows that whole yolk platelets, with a very low endogenous activity at low salt concentrations, acted immediately upon added exogenous substrate. YP_A, with the ghost protein removed, had a slightly greater endogenous activity, but a much lower activity upon phosvitin. The isolated ghost fraction was highly reactive against phosvitin. At high salt concentrations, the solubilized whole yolk had a greatly increased endogenous activity, but YP_A activity increased to a much lesser extent. With repeated precipitations, YP_A lost all of its activity against phosvitin and much of its endogenous activity (Nass, 1961).

(3) Table II presents data on the effect of increasing purification of the ovarian fractions upon their PPPase activity. The pigmented fraction retained a constant activity after several washings, while the small and large yolk fractions lost activity with each washing. The ratios of phosphate hydrolysis at low and high salt con-

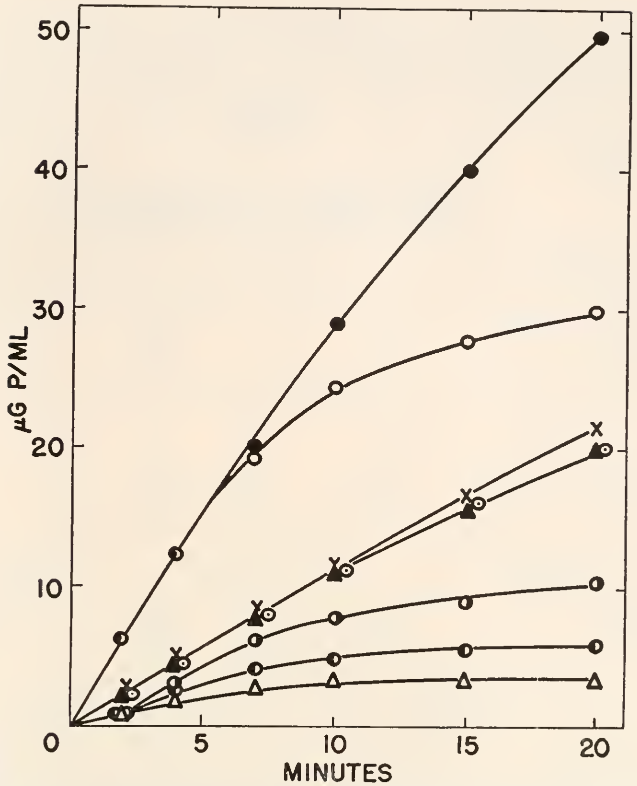

FIGURE 1. Rates of PPPase activity of yolk platelets and YP_A, incubated in 0.1 *M* and 0.4 *M* NaCl, with or without added phosvitin (10 m*M* P). Filled circle: yolk platelets + phosvitin in 0.4 *M* NaCl. Open circle: yolk platelets in 0.4 *M* NaCl. X: YP_A + phosvitin in 0.4 *M* NaCl. Filled triangle: YP_A in 0.4 *M* NaCl. Dotted circle: yolk platelets + phosvitin in 0.1 *M* NaCl. Circle, right half filled: YP_A + phosvitin in 0.1 *M* NaCl. Circle, left half filled: YP_A in 0.1 *M* NaCl. Open triangle: yolk platelets in 0.1 *M* NaCl. Incubated at pH 4.9, 28° C.

centrations show that most of the activity of the large yolk was due to its action on phosvitin in low salt but to its endogenous activity when the yolk was solubilized (see also Figure 1).

Fractionation of ovarian homogenates indicated that only the large and small yolk granules had any endogenous P_i released. Ghosts from the yolk platelets, the pigmented fractions and the supernatant proteins had no endogenous activity (Nass, 1961).

(4) The phosphorus/nitrogen ratio of yolk was 0.14, while the ratio of small yolk was 0.17, ghosts 0.21, and the pigmented fraction 0.28. YP_B fractions had somewhat lower ratios (0.10–0.12) from Shumway (1940) stage 1 through stage 20. Stage 25 YP_B had a distinctly lower ratio (0.08), probably related to new proteins being formed and precipitating with the euglobulin fraction (*e.g.* actomyosin). The increasing PPPase activities of the ovarian fractions against phos-

TABLE II

Comparison of total activities of ovarian fractions

Exp. No.	Fraction	Incubated in 0.1 M NaCl		Incubated in 0.4 M NaCl	
		(a) Ratio of total activity of fractions against phosvitin minus endogenous activity*	(b) Ratio of the sum of endogenous and phosvitin activities	(a)	(b)
1	Unwashed fractions				
	Pigment-supernatant	1.0	1.0		
	Pigmented small yolk	1.7	1.8		
	Large yolk	3.9	4.7		
2	"Cleaner" fractions				
	Pigment-supernatant	1.0	1.0		
	Pigmented small yolk	1.0	1.0		
	Large yolk	1.9	2.1		
3	"Cleanest" fractions				
	Pigment-supernatant	1.0	1.0	1.0	1.0
	Small yolk	0.7	0.7	0.5	0.7
	Large yolk	1.3	1.4	0.7	1.5

* The total activity of the pigment-supernatant is arbitrarily set at 1.0.

vitin (Figures 2 and 3) were found to parallel their P/N ratios. The exceptional behavior of YP_B having a high PPPase activity against phosvitin (but a lower P/N ratio than yolk) relates to the loss of P_i from YP_B during its preparation. The activity of this fraction may be due to its greater pigment contamination which is evident by the grayness of the preparation, although the P/N ratios would not be markedly altered because of the predominance of the yolk protein.

C. *Properties of PPPase*

1. *Rate of phosphoprotein hydrolysis.* Figure 4 represents the rates of phosvitin hydrolysis by pigment extract. The reaction velocity was linear for 15 minutes, comparable to the rates found for the other fractions discussed previously. (Figs. 2 and 3).

The velocities of P_i release and of protein solubilization from heated yolk by the action of the enzyme are shown in Figure 5. The experimental procedure involved incubation of heated yolk in concentrated salt solutions buffered to pH 4.9. An aliquot was removed for P_i analysis and another aliquot added to 9 volumes of ice-cold water. The precipitate that formed was centrifuged and an aliquot of the supernatant analyzed for protein.

The specific activity of the extract in the presence of optimal concentrations of heated yolk and phosvitin was not higher than with either substrate alone, thus providing evidence for the action of a single enzyme.

Figure 5 shows that the linear rate of phosphate release is only maintained as long as the rate of protein solubilization is linear. In addition, no activity of the extract against heated whole yolk in 0.1 *M* NaCl was found although ghost-free YP_A was solubilized and dephosphorylated under these conditions. These results suggest that disruption of the yolk platelet organization is essential for the action of the enzyme.

Non-protein nitrogen did not increase during incubations with phosphoprotein, indicating that no proteolysis had taken place.

2. *Enzyme concentration.* The results summarized in Figure 6 show that a direct relationship exists between protein concentration and velocity with both phosphoprotein substrates tested. Variations in the salt concentration had little

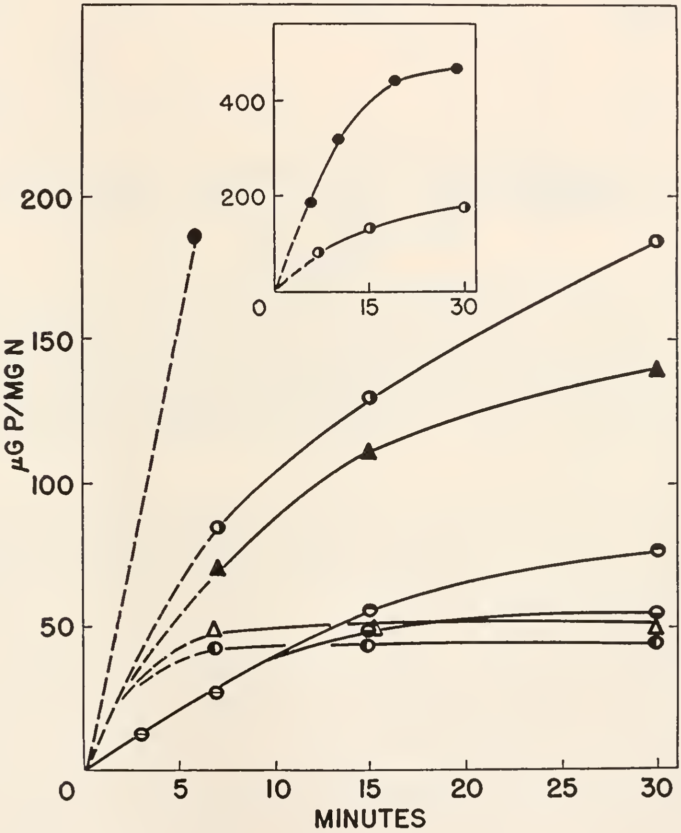

FIGURE 2. Rates of PPPase activity of centrifugal fractions of ovarian homogenates with or without added phosvitin. All incubations in pH 4.9 buffer, 0.4 *M* NaCl, 28° C. Filled circle: pigment + phosvitin. Circle, right half filled: small yolk + phosvitin. Filled triangle: whole homogenate + phosvitin. Circle, upper half filled: large yolk + phosvitin. Circle, lower half filled: large yolk endogenous activity. Open triangle: whole homogenate endogenous activity. Circle, left half filled: small yolk endogenous activity. Inset compares pigment and small yolk activities against phosvitin throughout the 30-minute incubation.

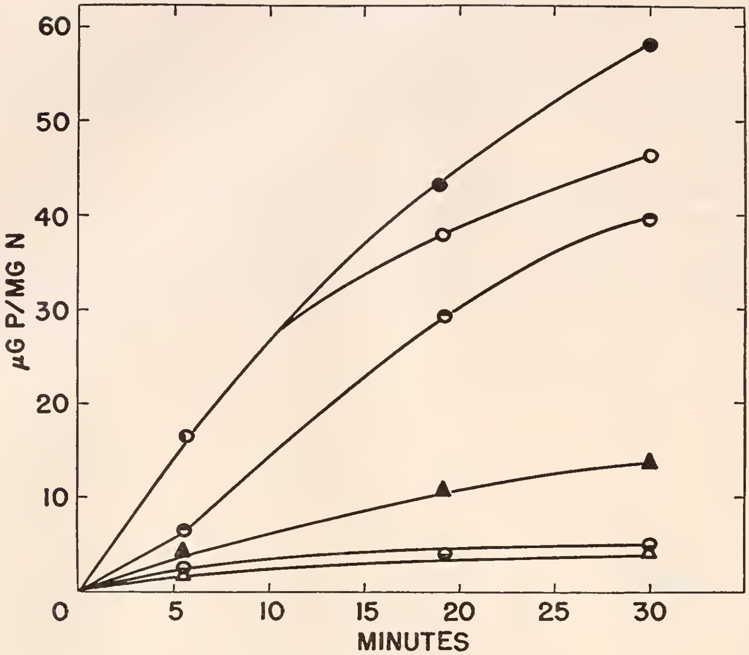

FIGURE 3. Comparison of enzymatic activity of yolk platelets and YP_B in 0.1 *M* and 0.4 *M* NaCl, with or without the addition of phosvitin. YP_B was mostly dephosphorylated prior to incubation, as described in text. Filled circle: yolk platelets + phosvitin in 0.4 *M* NaCl. Open circle: yolk platelet endogenous activity in 0.4 *M* NaCl. Circle, upper half filled: YP_B + phosvitin in 0.4 *M* NaCl. Filled triangle: yolk platelets + phosvitin in 0.1 *M* NaCl. Circle, lower half filled: YP_B endogenous activity in 0.4 *M* NaCl. Open triangle: yolk platelets, endogenous activity in 0.1 *M* NaCl.

effect when the enzyme was incubated with water-soluble phosvitin, whereas heated yolk platelets were an effective substrate only at high salt concentrations. When heated YP_A was used as a substrate, a high hydrolytic rate was found in both weak and strong salt solutions.

3. *Substrate concentration.* Phosvitin, a yolk protein with 9.7% of its weight as phosphorus and a P/N ratio of 0.8 (Mecham and Olcott, 1949), was dissolved in buffer, and the molarity of phosphorus was used as the basis for the substrate concentration curves. Yolk with a P/N ratio of 0.14 required over five times as much protein to reach the same phosphorus concentration. It is apparent from Figure 7 that the phosvitin concentration is nearly optimal at 7 m*M* phosphate, while heated yolk does not approach an optimum until 20 m*M* phosphate. A Lineweaver-Burk analysis (Lineweaver and Burk, 1934) resulted in a Michaelis constant of 3.6 m*M* of phosvitin phosphorus.

Pigment extract had no activity after 15 minutes of incubation against 10 m*M* P concentrations of β-glycerophosphate, disodium phenyl phosphate, and ATP.

4. *Effects of pH.* The results of variations of the pH upon the PPPase activity of pigment extracts with phosvitin as substrate are shown for two temperatures in

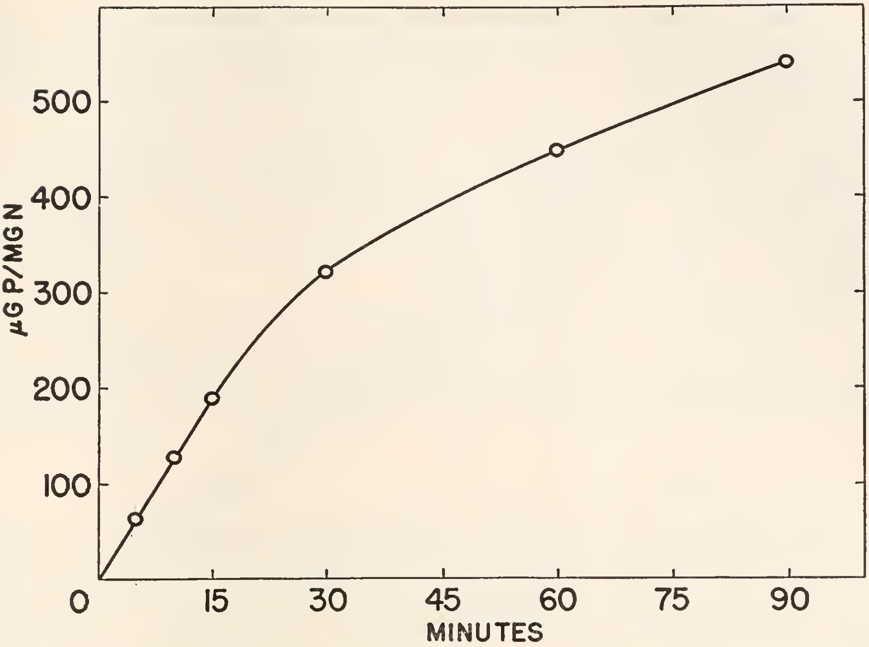

FIGURE 4. Rate of phosphate hydrolysis from phosvitin (10 m*M* P) by pigment extract. Incubated at pH 4.9, 28° C.

Figure 8. The pH curves are very similar to those reported for the endogenous activity of yolk platelets (Nass, 1956) except for a shift of the pH optimum from pH 5.0 in the latter to pH 5.4 with phosvitin.

5. *Effects of temperature.* The activity of the extract against heated yolk in 0.4 *M* NaCl was tested at various temperatures. The temperature optimum for a 10-minute incubation period was found to be at about 50° C., which is similar to that found previously for endogenous yolk activity (Nass, 1956). A sharp decline in the activity occurred from approximately 55° to 65° C. Arrhenius plots of these data and those of a similar experiment with phosvitin as a substrate are shown in Figure 9. Both substrates gave very similar plots with discontinuities at 26° C.

6. *Ionic effects.* Many references have been made throughout this section about the effects of strong salt solutions (0.4 *M* NaCl) and of weak salt solutions (0.1 *M* NaCl). In summary, the activity of the extract upon yolk platelets is minimal in 0.1 *M* NaCl but much greater in 0.4 *M* NaCl. The effects of the ionic strength of the medium on the action of the extract upon phosvitin are very small between 0.4 and 0.1 μ, but a 20% decrease of activity was measured when the ionic strength was lowered to 0.02 μ.

Ca^{++} and Mg^{++} completely inhibited the P_i release at 10^{-1} *M* concentrations but were without effect at lower concentrations. Cu^{++}, Hg^{++}, Mn^{++}, Fe^{+++}, and Al^{+++} completely inhibited the P_i release at 10^{-1} *M* concentrations, and exhibited inhibitions of 75% and 15% at 10^{-2} *M* and 10^{-3} *M*, respectively. Most of the effects were a resultant of precipitation of the reactants at high ionic concentrations.

7. *Inhibitors.* Both the endogenous and exogenous activities of yolk fractions were markedly inhibited by ammonium molybdate, suggesting that both reactions were enzymatically similar. The Lineweaver-Burk plot of molybdenum inhibition of PPPase extracted from the pigment fraction showed this polyanion to be a non-competitive inhibitor of the enzyme. The ammonium molybdate was observed to precipitate a very small quantity of non-TCA-precipitable material when added to an incubation mixture. The clouding that was usually found when molybdate was added during the Fiske-Subbarow procedure was not observed when this reagent was added to molybdate-inhibited incubation mixtures after TCA precipitation. Inhibition was 50% at 10^{-4} *M* molybdenum.

Other reagents that were reported to be activators and inhibitors of mammalian PPPase (Feinstein and Volk, 1949; Norberg, 1950; Sundararajan and Sarma, 1954; Paigen, 1958), including thioglycollic acid, desoxycholate, ascorbic acid, hydrogen peroxide, and phenanthroline, did not affect frog PPPase at concentrations of 10^{-2} *M*. Oxalate, iodoacetate, cyanide, and fluoride were equally ineffective.

D. *Changes in PPPase activity during development of frog embryos*

Figure 10 shows that the amount of YP_B protein per embryo decreased sharply after stage 20 (hatching). Pigment residues, obtained from ovaries and embryos at different stages of development, were incubated individually with YP_B of stage 2, stage 4, stage 10, stage 20, and with phosvitin. The activities of each residue

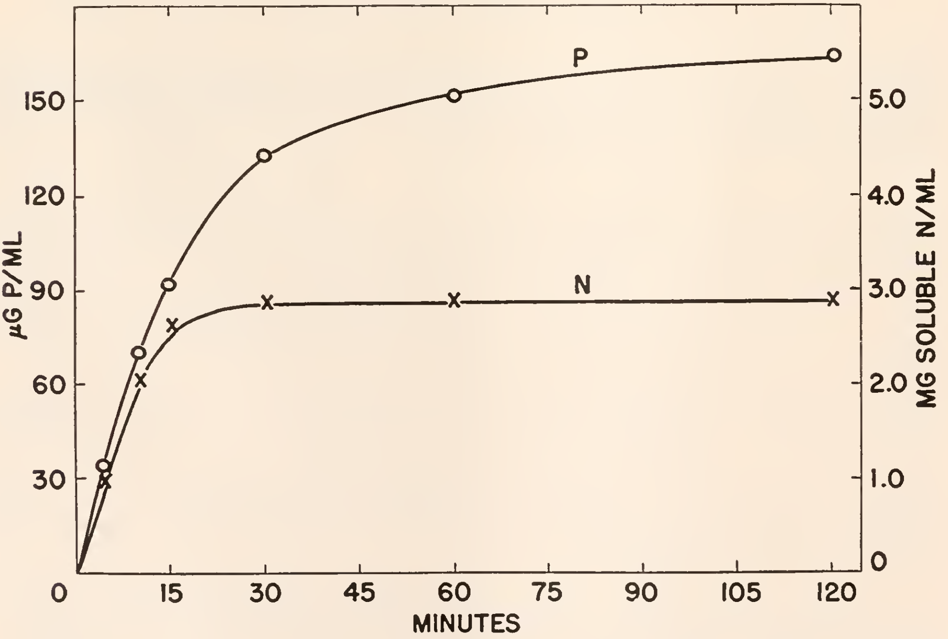

FIGURE 5. Rate of phosphate hydrolysis and protein change from a water-precipitable to a water-soluble state by the action of pigment extract upon heated yolk platelets in 0.4 *M* NaCl, pH 4.9, 28° C.

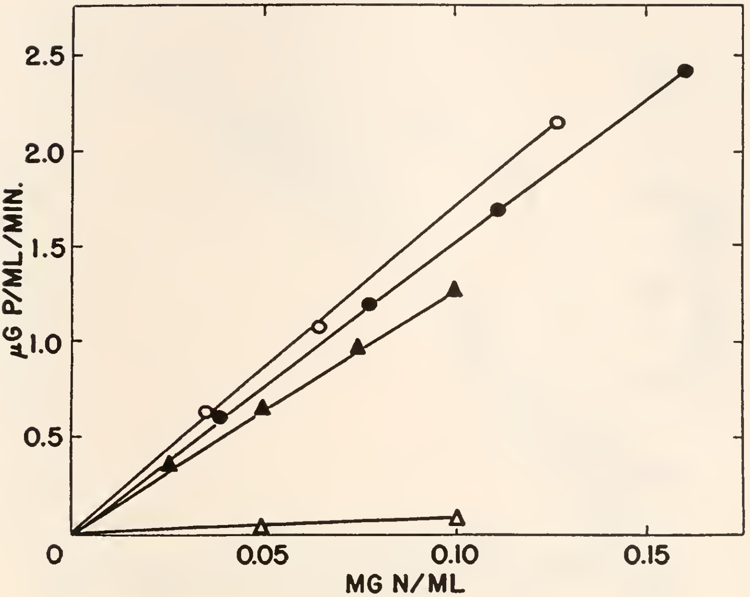

FIGURE 6. The proportionality of the reaction rate to enzyme concentration. Substrates: Open circle: heated yolk platelets (20 m*M* P) in 0.4 *M* NaCl. Filled circle: phosvitin (10 m*M* P) in 0.4 *M* NaCl. Filled triangle: phosvitin in 0.1 *M* NaCl. Open triangle: heated yolk platelets in 0.1 *M* NaCl. Incubation period 15 minutes, pH 4.9, 28° C.

against all of these substrates were similar, and the average value obtained from the incubation of each residue with the five substrates is reported in the figure. Although ovarian pigment had a specific activity of 21 μg. P/mg. N/min., the residues after fertilization had very low activities until stage 17 (tailbud), when the activity sharply increased to the value found for ovarian pigment. The decline of the activities of the residues after hatching paralleled the decline of YP_B extractable from embryos. It should be re-emphasized that the activities of the residues declined after stage 20 when YP_B of stage 2 through stage 20 as well as phosvitin was used as substrate, and therefore the decline was not a result of the smaller quantities of substrate present in embryos after stage 20.

DISCUSSION

The contradictory reports on the localization of the enzyme phosphoprotein phosphatase in the frog egg appear to be a result of the different preparatory techniques, which lead to variable amounts of adsorption artifacts or to non-enzymatic dephosphorylation of the yolk proteins during preparation. Yolk proteins prepared by homogenization and extraction of ovaries at high ionic strength are largely dephosphorylated during the preparation and therefore lose their characteristic euglobulin properties (Table I). This property is not exhibited by yolk proteins prepared by similar extraction techniques of whole yolk platelets. No water-soluble livetin is found when isolated, clean yolk platelets are solubilized and re-precipitated. This result is in agreement with that reported by Ringle (1960).

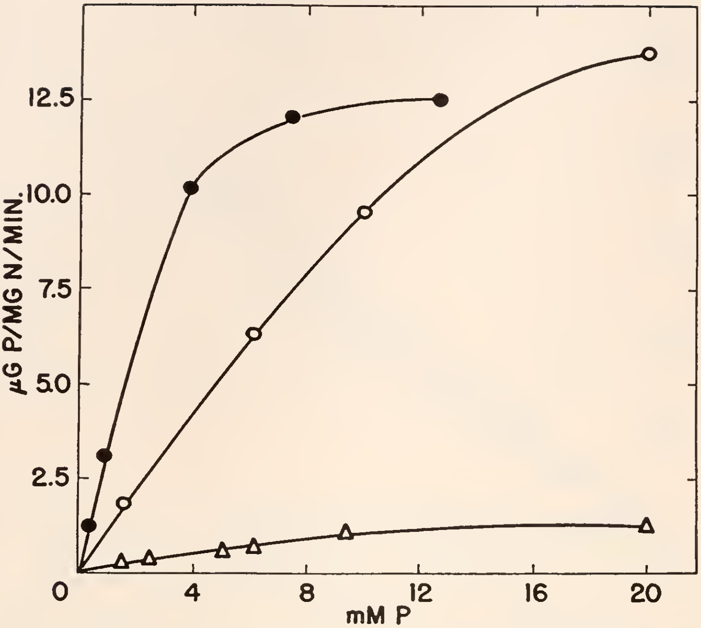

Figure 7. Effect of substrate concentration upon enzyme activity. Filled circle: phosvitin in 0.4 *M* NaCl. Open circle: heated yolk platelets in 0.4 *M* NaCl. Open triangle: heated yolk platelets in 0.1 *M* NaCl. Incubation period 15 minutes, pH 4.9, 28° C.

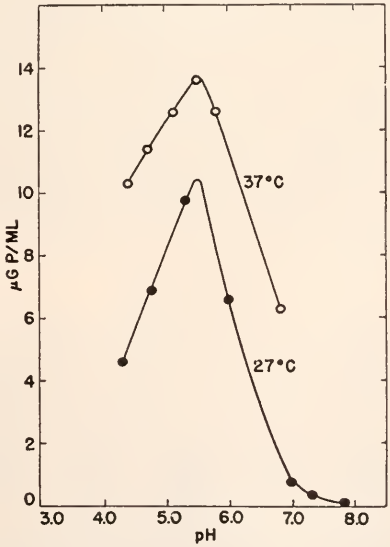

Figure 8. The pH dependence of phosphate release. Phosvitin as substrate. Incubation period 10 minutes, 0.05 *M* acetate buffers.

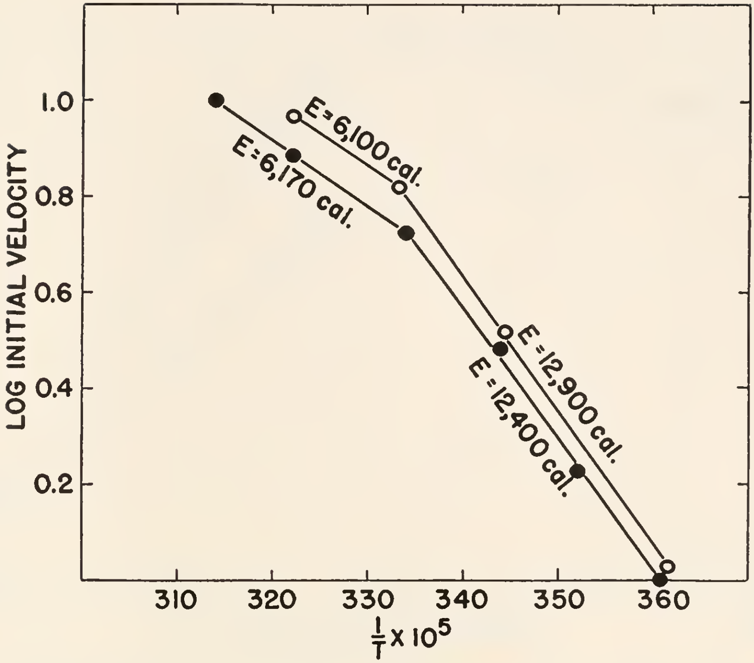

FIGURE 9. Arrhenius plots for PPPase reaction. Upper curve: phosvitin (10 m*M* P) as substrate. Lower curve: heated yolk platelets (20 m*M* P) as substrate. Incubations in 0.4 *M* NaCl, pH 4.9. Incubation period, 10 minutes.

The heavily contaminated YP_B preparations also had a higher RNA phosphate content than clean yolk fractions (Table I), and it is suggested that the small quantities of RNA measured in all the yolk fractions may be due to adsorption of nucleic acids.

Properties of yolk, such as P/N ratios, the presence or absence of enzymes, the number of electrophoretic, ultracentrifugal and serological components, may need re-examination because of the instability of the material prepared for characterization. The wide variations in P/N ratios reported (0.05 to 0.14) (McClendon, 1910; Fauré-Fremiet and du Streel, 1921; Lawrence *et al.,* 1943; Panijel, 1950; Gross and Gilbert, 1956; Ringle, 1960) may be related to whether the yolk was carefully cleaned before solubilization and re-precipitation or was first extracted and then re-precipitated. The presence of PPPase reported by L. J. Barth (1956) in yolk extracts may be correlated with the adsorption of pigment and with non-enzymatic dephosphorylation. The variable number of electrophoretic, serological and ultracentrifugal components reported for yolk may also be correlated with the dissociation of the yolk proteins following dephosphorylation. Barth and Barth (1954) estimated that the non-phosphoprotein component of yolk made up approximately 90% of the total protein of the yolk. The possibility that part of their yolk preparations was dephosphorylated during preparation is suggested by the results of this study. Thus, the YP_B preparations were found to be unstable and dephos-

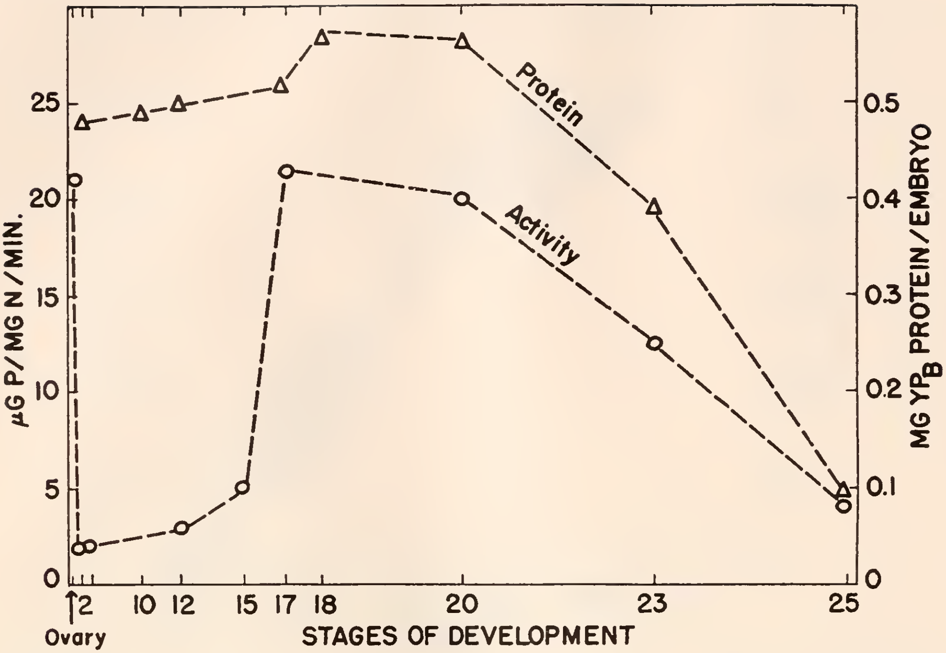

FIGURE 10. Changes in the specific activity of PPPase in pigment residues during embryonic development, relative to the changes in salt-extractable, water-precipitable protein. Abscissa: stages of development (Shumway) superimposed upon an hour of development scale. Activity values are average values, as described in the text. Incubation period, 10 minutes. Buffer, pH 4.9, containing 0.4 *M* NaCl; 28° C.

phorylated during preparation. Further, the YP_B is largely acid-soluble while YP_A is mostly acid-precipitable. Finally, the required pH for solubilization of yolk (pH 11.5) will partly dephosphorylate the yolk non-enzymatically (Flickinger, 1956). The ultracentrifugal and electrophoretic analyses of Schjeide *et al.* (1955) involved a concentrated salt extraction from a pigment-yolk mixture and thus may contain large quantities of water-soluble dephosphorylated vitellin (livetin). Mixing of a preparation of livetin with a preparation of vitellin (YP_B of this report) gave a single electrophoretic peak. The small 6S component (phosphoprotein) and the much larger 11S component (non-phosphoprotein) found by ultracentrifugal analyses (Flickinger and Schjeide, 1957) may also be related to partial dephosphorylation of the preparations prior to analysis. That livetin had other heavier components, 16S and 19S (Schjeide *et al.*, 1955), is suggestive of the report by Gross and Gilbert (1956) that calcium solubilized yolk aggregates with time and thereby changes its sedimentation from 11S to 50–60S. The immunological analyses of Ringle (1960) indicated that the number of apparent protein antigens present in yolk can be changed by altering the salt concentration, the ionic species, or by freezing and thawing. The observations described suggest that the yolk protein is very unstable after it is solubilized, and caution must be used in interpretation of the results of analyses of these preparations.

The conclusions of Ringle (1960) based on micromanipulation, serological and electron microscopic techniques, that the ghost material is not a normal feature of the yolk platelet, are in agreement with the enzymatic studies performed in the present investigation. The ghost protein had a high activity against phosvitin and probably accounts for the activity previously reported to be present in the yolk (Harris, 1946; Panijel, 1950; Nass, 1956). Thus, whole yolk platelets in dilute salt solutions acted immediately on phosvitin, although they had little endogenous activity, while solubilized yolk had no additional activity in the presence of phosvitin (Fig. 1). The P/N ratios of yolk, ghost proteins and pigment further suggest the closer relation of the gray ghost material to the black pigment fraction. The small yolk platelets, having a greater surface/volume ratio, would absorb more of the pigment material, which would thus account for the greater PPPase activity reported (Panijel, 1950) for this fraction as compared with the large yolk granules. Other evidence supporting the view that the ghost surface of the yolk is largely a pigment contaminant and not a regular feature of yolk includes the finding that removal of the ghost material decreased the endogenous activity of the yolk protein and eliminated its action against phosvitin. Further, Table II shows that although the yolk fractions contained the greater total activity, the activity declined markedly with repeated washing of the yolk fractions, but washing did not alter the activity found in the pigment fraction. The reported inability to standardize the enzyme activity of different yolk preparations by measuring the protein content of samples (Nass, 1956) undoubtedly reflects variable quantities of pigment adsorbed to different yolk preparations.

The properties of the enzyme have not previously been studied in preparations free of endogenous activity and in solution. The reported characteristics of the endogenous activity (Nass, 1956) are similar to the properties measured with exogenous substrates and with the enzyme in solution. The equivalent activity of pigment extract on phosvitin at low and high ionic strengths indicates that the salt concentration is a factor of importance only in the solubilization of the yolk proteins and not in activation of the enzyme. The reported activation of the enzyme by Ca^{++} (Flickinger, 1956) may be related to the solubilization of the yolk that is a prerequisite for enzymatic activity against yolk. No activators were found and only molybdate ions out of many agents tested acted as an inhibitor. Except for the mammalian PPPase inhibition by molybdate, the properties of frog PPPase do not resemble any of those reported for the mammalian enzyme (Sundararajan and Sarma, 1954, 1959; Paigen, 1958). The amphibian enzyme is more substrate-specific, no enzymatic action being observed under standard incubation procedures when β-glycerosphosphate, disodium phenyl phosphate or ATP are used as substrates (Nass, 1961).

The experiments reported on the embryonic YP_B preparations show that this material is much more stable than ovarian YP_B. The preparations are noticeably cleaner than ovarian YP_B, and they have negligible endogenous activity. Figure 10 shows that at fertilization the pigment residues lose all their PPPase activity against all the phosphoprotein substrates and that the activity of the residues did not increase again until late gastrulation. The activity reached a maximum at tail-bud stage, where the specific activity was found to be equivalent to that found for the ovarian pigment fraction. The specific activity of the pigment residues dropped sharply after hatching, paralleling the decline in the quantity of yolk. The water-soluble protein of the frog embryo (which is probably dephosphorylated yolk) increases at neurulation (Gregg and Ballentine, 1946; M. Nass, 1961), which is the approximate time at which PPPase activity increases in the embryo. Fertilization thus apparently inhibits the enzymatic activity found in the pigment granule fraction of the unfertilized egg, and concomitantly the YP_B preparations are cleaner and more stable.

There is clear agreement that yolk utilization is correlated with differentiation and that the most rapidly differentiating areas are the locations for the earliest yolk solubilization (Bragg, 1939; Flickinger, 1949). Flickinger (1956, 1957) and Holtfreter (1947) have maintained that yolk solubilization is of primary importance in inducing neural structures, since ventral explants can be neuralized by treatments which solubilize the yolk proteins. The localization of PPPase in the pigment fraction (probably composed mostly of cortical material) adds support to these views and to the cortical-vitellin gradient hypothesis of Dalcq and Pasteels (1937). Pasteels (1951) suggested that at fertilization the precursor of the active principle of the organization center can be displaced, but after the gray crescent is formed the precursor is "fixed" in the cortical areas and cannot be displaced by centrifugation. This may be compared with the high activity found for PPPase in the homogenized unfertilized egg and with the loss of activity of the pigmented fraction shortly after fertilization. The "fixation" of the cortex would then correspond to the loss of activity of the cortical pigment fraction.

The first area of contact of the cortex with the yolk occurs at the dorsal lip of the blastopore. Although Figure 10 does not indicate more than a very sight rise in the early activity of PPPase, it may become more apparent if experiments were performed upon dissected fragments of this cortical area. The "organizer" action of the dorsal lip could then be interpreted as freeing the yolk-hydrolyzing mechanism, which would provide the necessary materials for neuralization. Experimental neuralization would then involve supplying hydrolyzed yolk proteins to cells which have the capacity but lack the material for neuralization.

Curtis (1960) has shown that the cortical material of cleaving *Xenopus* eggs possesses morphogenetic properties which may be transferred with it; however, the cortical material of the unfertilized egg inhibits development when grafted to a fertile egg. This is also suggestive of a change in the cortex that occurs upon fertilization. Mezger-Freed (1953) studied PPPase activity in the frog and reported that dorsal portions of gastrulae had a greater activity per unit of nitrogen but a lower total activity than ventral fragments. Table II also shows the pigmented fraction to have the greatest activity per unit of nitrogen, but the yolk fraction (mostly vegetal material) had the greatest total activity in unfertilized eggs. These results suggest that the enzyme is located in the dorsal part of the egg cortex, but homogenization disrupts the spatial orientation and thus allows for greater enzyme-substrate interaction.

The localization of the enzyme in the pigment fraction also suggests that the controlling mechanism of yolk utilization is the morphogenetic movement which brings the cortex in contact with the yolk. The leading edge of the invaginating cortex is the area of most active differentiation, and it is of interest that this area is continually in contact with the yolk.

I am grateful to Professor Paul R. Gross for his sponsorship and encouragement throughout this investigation. It is a pleasure to acknowledge the hospitality and the facilities provided by the laboratories at University Heights, New York University, and the Marine Biological Laboratory at Woods Hole, Mass. I am also grateful to Dr. Margit Nass for donating the embryo material, for many suggestions and for her criticism of the manuscript.

SUMMARY

1. The phosphoprotein phosphatase activity of various centrifugal fractions and extracts of homogenates of frog ovaries and embryos was studied in the presence and absence of added phosphoprotein substrates and as a function of a number of environmental variables, including incubation time, pH, temperature and ionic concentrations.

2. Yolk prepared in low ionic strength media was far more stable than yolk extracted from whole homogenates with concentrated salt solutions followed by precipitation by dilution or dialysis. The latter fraction lost its characteristic euglobulin properties during preparation, and this was correlated with the loss of phosphoprotein phosphorus from this material.

3. The rate of endogenous P_i release from yolk platelets was found to be a function of the rate of solubilization of the platelets, and the rate of P_i release was linear only as long as the rate of increase of water-soluble protein was linear.

4. The pigment-containing centrifugal fraction was found to have the highest PPPase activity when ovarian fractions were incubated in the presence of added phosphoproteins. Evidence is presented that the activity found in other centrifugal fractions and extracts is a result of varying degrees of pigment contamination.

5. It was found possible to study the enzyme characteristics of PPPase with the enzyme in solution and in the absence of endogenous substrate.

6. Yolk extracts from embryos were far more stable after re-precipitation by water than similarly prepared ovarian extracts. The enzyme activity of the pigment-containing fraction was found to drop precipitously after fertilization, slowly increase from gastrulation to neurulation and rise to a maximum at tailbud. The activity declined after hatching.

7. The possible sources of the discrepancies in the literature concerning the site of PPPase and the composition of yolk proteins are discussed. The significance of the localization studies and the variations in enzyme activity found during embryogenesis is discussed in relation to the morphological changes occurring during that period.

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OXYGEN-HEMOCYANIN RELATIONSHIPS IN THE LAND CRAB, CARDISOMA GUANHUMI[1]

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Relatively little quantitative information is available concerning the *in vivo* oxygen levels in the blood of the decapod Crustacea. Investigations upon three decapods, the spiny lobster, *Panulirus interruptus,* the American lobster, *Homarus americanus,* and the sheep crab, *Loxorhynchus grandis,* indicated that the total oxygen content of the blood was low and that the oxygen tension in both pre-branchial and post-branchial blood samples was not over 12 mm Hg. These low oxygen tensions resulted from the failure of the hemocyanin in the above forms to become saturated with oxygen as the blood passed through the gills. The hemocyanin was found to be responsible for most of the oxygen transported to the tissues since the blood, operating at very low oxygen pressures, could carry very little in physical solution (Redmond, 1955). Zuckerkandl (1957a, 1957b), on the other hand, found in the crab, *Maia squinado* (=*Maja*), that during certain stages of the molt cycle the hemocyanin might disappear completely, and at this and other times the oxygen tension in the post-branchial blood was considerably higher than in the previously mentioned three species. The present study of the land crab, *Cardisoma guanhumi* Latreille, was undertaken to add to the knowledge of blood-oxygen relationships in living decapods and to extend these observations to an organism from an environment unlike that of the above species.

OXYGEN EQUILIBRIUM CURVES

Specimens of *Cardisoma* were collected from two locations, the Port Royal area of Jamaica, W. I., and Coral Gables, Florida. Blood samples to be used in the determination of oxygen equilibrium curves were withdrawn from the bases of the walking legs by means of 5-ml. hypodermic syringes. The blood was allowed to clot and was then expressed through cheese-cloth. Only freshly drawn blood was used. When necessary, the pH of the blood was adjusted with borate or Tris buffers; otherwise, the blood was untreated. Standard borate solutions were mixed one-to-one with the blood samples and were used in a first series of measurements. In later measurements two concentrated solutions of Tris in sea water (1 gram Tris: 10 ml. water), adjusted with HCl to pH 8.5 and pH 6.7, were added to the blood in small quantities to achieve various levels of pH without appreciably changing the concentration of the hemocyanin. The pH of the blood samples was taken after the blood had been equilibrated with air, immediately prior to the

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determination of the oxygen equilibrium curves. Much of the diffusible carbon dioxide would have been lost at this time; however, the possibility exists that additional carbon dioxide loss at low pressures may have resulted in a final pH somewhat higher than that recorded originally. Comparison of the oxygen equilibrium curves of undiluted blood with blood of the same pH to which these buffers had been added, indicated that, at least at normal blood pH, the buffers did not noticeably alter the curve.

Oxygen equilibrium curves were determined by the vacuum pump-spectrophotometric method described previously (Redmond, 1955), or by a modification of this procedure similar to that of Jones (1954). In the original procedure a cylindrical cuvette containing the blood was connected by pressure tubing to a vacuum pump. This connection was maintained throughout the determination, and equilibration was achieved by gently shaking the cuvette by hand. In the modified procedure the cuvette is replaced by one or more small tonometers which can be sealed and disconnected from the pressure tubing after the desired internal pressure has been reached. One end of the tonometer consists of an exchangeable cuvette which can be placed directly in the well of a Coleman Jr. spectrophotometer. Equilibration is effected by placing the tonometer in a slowly moving (62 cycles/min.) reciprocal shaker. The latter method has several advantages. In addition to not requiring the constant attention of the investigator during equilibration, more than one curve may be determined simultaneously. Longer periods of equilibration are required, but, since the shaking is slower and more controlled, less cloudiness due to protein denaturation occurs. Another important factor is that, because the tonometer is sealed, it is easier to maintain consistent low pressures. Depending upon the size of the cuvette portion of the tonometer, 0.5 to 3.0 ml. of blood were required for each curve. In the present series of experiments, optical density readings were made at 575 millimicra, the wave-length of maximum absorption for the oxyhemocyanin of *Cardisoma*. Temperatures were maintained at ±0.5° C. At pH 7.5 and 25° C. the hemocyanin of *Cardisoma* becomes half-saturated with oxygen at a partial pressure of about 3.5–4 mm. Hg (Figs. 1 and 2). This is an unusually high oxygen affinity for a crustacean hemocyanin. Only one other crustacean hemocyanin, that of the crayfish, *Procambarus simulans,* has been reported to have as low a half-saturation pressure. Latimer (1961) found this hemocyanin to become half-saturated at 3.5 mm. O_2 at 25° C. and pH 7.7. The possible significance of this high affinity will be discussed later.

Figure 1 illustrates representative oxygen equilibrium curves and Figure 2 indicates how the pressure of half saturation varies with changing pH. Both figures show that this pigment, like all other known crustacean hemocyanins, possesses a normal Bohr effect. For purposes of comparison the extent of this shift in half-saturation with pH can be expressed by the equation

$$\phi = \Delta \log p_{50}/\Delta \mathrm{ph} \qquad \text{(Wyman, 1948).}$$

For the interval pH 7.3 to pH 7.8, at 25° C., *Cardisoma* hemocyanin has a value of $\phi = -0.75$. Since there are so few comparable measurements it is difficult to judge whether or not this is a normal figure for decapod Crustacea. For the spiny lobster, *Panulirus interruptus*, $\phi = 0.30$ (Redmond, 1955). What little evidence exists suggests that the values of ϕ for crustacean hemocyanins probably lie in the

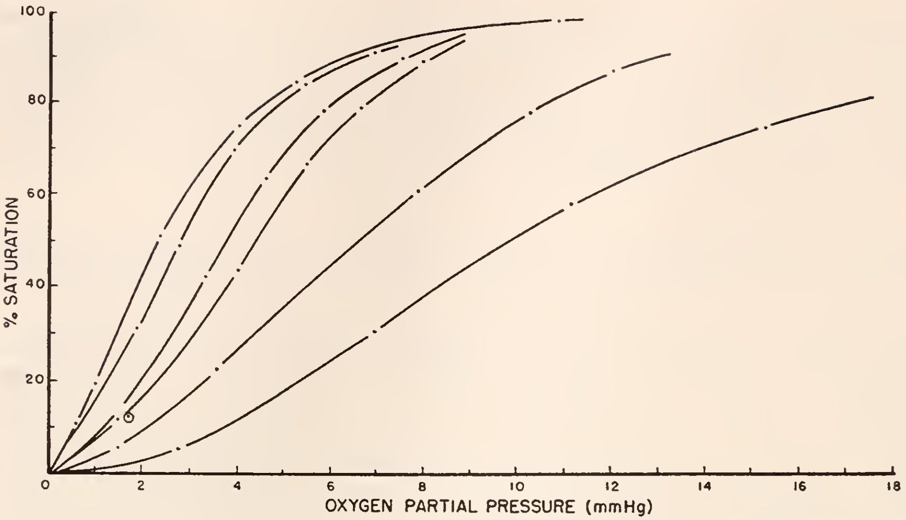

FIGURE 1. Oxygen equilibrium curves for the hemocyanin of *Cardisoma guanhumi* at 25° C. The pH at which each curve was determined is, from left to right, 7.90, 7.70, 7.54, 7.41, 7.30, 7.01.

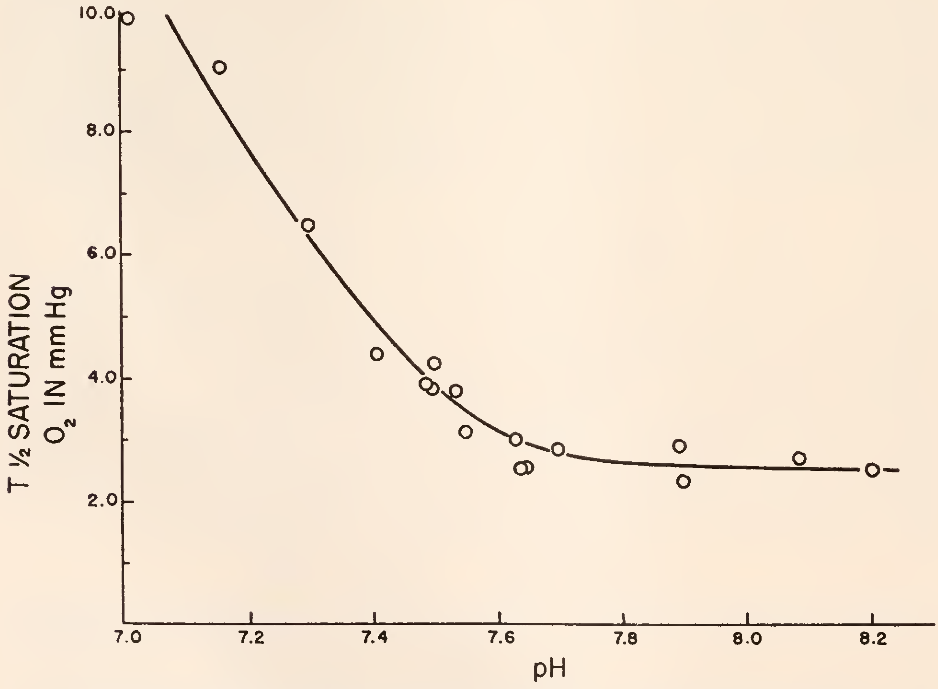

FIGURE 2. The effect of pH upon the pressure at which the hemocyanin of *Cardisoma guanhumi* becomes half-saturated. 25° C.

Table I

Values of "n" for oxygen equilibrium curves of the hemocyanin of Cardisoma guanhumi at normal pH

pH	"n"
7.41	2.81
7.50	2.69
7.54	2.50
7.55	2.73
7.63	2.31
7.64	2.78
Av. 7.55	2.64

range of those found for vertebrate hemoglobins; horse −0.60 (St. George and Pauling, 1951), surf duck −0.58, western grebe −0.45 (Manwell, 1958), mackerel, *Scomber scomberus,* −1.02 (Nicol, 1960). The latter values are calculated for the pH range 7.0–7.5. The crustacean hemocyanins would have appreciably higher values over this range, but since the pH of their blood is usually near 7.5, the range 7.3–7.8 would appear to be of more physiological interest.

The sigmoid shape of the oxygen equilibrium curves of *Cardisoma* hemocyanin indicates that interaction occurs among the oxygen-combining sites of the hemocyanin molecule. Plotting the oxygen equilibria data in the form of log p vs. log (y/100 − y), where p is the partial pressure of oxygen and y is the corresponding per cent saturation, yields a line whose slope, "n," is an approximation of the extent of interaction (Klotz, 1954). Six values of "n" (Table I) at normal blood pH average 2.64. Thus a considerable degree of positive facilitation occurs in the binding of oxygen by the different sites of the *Cardisoma* hemocyanin molecule.

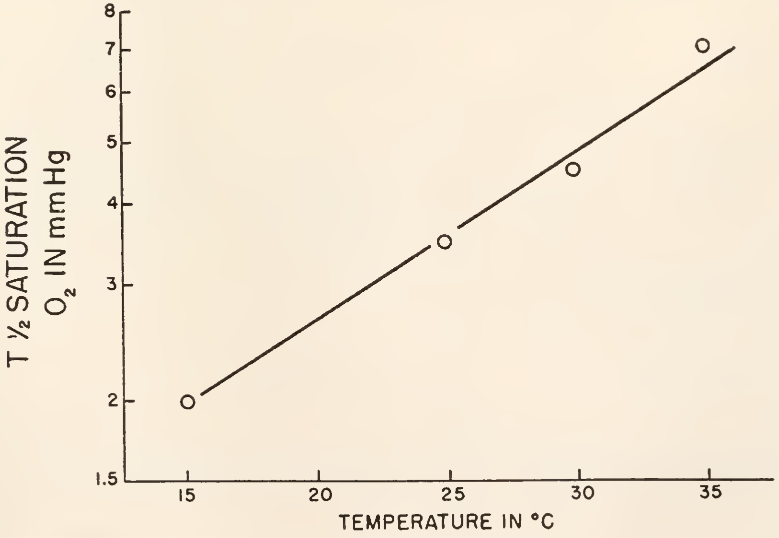

Figure 3. The effect of temperature upon the pressure at which the hemocyanin of *Cardisoma guanhumi* becomes half-saturated; pH 7.53.

Figure 3 illustrates the effect of changing temperature upon the pressure of half-saturation. The slope of the graph is similar to that of several other hemocyanins (unpublished data), but, because of the high affinity of *Cardisoma* hemocyanin for oxygen, the absolute change in half-saturation pressure is small. At 15° C. the pigment becomes half loaded at 2 mm. Hg of oxygen, while at 35° C. this figure has increased only to 7 mm.

BLOOD-GAS ANALYSES

Direct analyses of oxygen and carbon dioxide in the blood were carried out by the Van Slyke constant volume manometric technique (Consolazio, Johnson and Marek, 1951). One-ml. samples of blood were used, with 5% KCN and 1 N lactic acid being used to free the oxygen and carbon dioxide, respectively (Redmond, 1955). Post-branchial blood was taken by means of a 2-ml. hypodermic syringe from the pericardium, pre-branchial blood from the ventral thoracic sinus. Table II summarizes the results of the oxygen analyses. These measurements were made at 29° C. on freshly captured Jamaican crabs, all of which were in stage C_4 of the molt cycle. The water of Kingston Harbor and the daytime air temperature were both 29° C. The oxygen capacity of the hemocyanin was approximated by subtracting, from the oxygen capacity of the whole blood, 90% of the amount of oxygen that would dissolve in sea water at 29° C. The per cent saturations of the hemocyanin in the pre-branchial and post-branchial blood samples were calculated

TABLE II

Oxygen levels in pre-branchial (V) and post-branchial (A) blood of Cardisoma guanhumi

Species	Oxygen capacity*		*In vivo* oxygen content								
	Whole blood Vol. % O_2	Hcy Vol. % O_2	Total vol. % O_2			Hcy % sat.		O_2 pressure** mm. Hg		% of total O_2 as oxy hcy	
			A	V	A-V	A	V	A	V	A	V
Cardisoma guanhumi	3.01	2.61	1.65	0.87	0.78	62	30	5.1	3.0	99	99
	2.55	2.15	1.75	0.99	0.76	81	35	6.9	3.4	99	99
	2.93	2.53	1.57	0.85	0.72	61	33	5.1	3.2	99	99
Average	2.83	2.43	1.66	0.90	0.75	68	33	5.7	3.2	99	99
*Panulirus interruptus****											
Average of nine specimens	1.99	1.53	0.82	0.35	0.46	54	22	7	3	96	97
*Loxorhynchus grandis****											
Average of five specimens	1.03	0.58	0.41	0.17	0.24	68	30	8	3	90	94
*Homarus americanus****											
Average of two specimens	1.31	0.86	0.44	0.18	0.26	49	20	5	2	95	95

* Oxygen content following equilibration with air at 29° C. at atmospheric pressure.

** Since the pH values of the blood samples were not determined, the calculations of post-branchial oxygen pressures from an oxygen equilibrium curve determined at pH 7.53 renders these pressures uncertain by approximately +1.5 to −1.0 mm. Hg. For reasons detailed in Redmond (1955), the A-V pH shift is believed not to be great enough to significantly affect these calculations.

*** The data for *Panulirus*, *Loxorhynchus*, and *Homarus* are taken from Redmond (1955).

by trial addition of the oxygen equilibrium and oxygen solubility curves until the sums were equal to the amounts of oxygen indicated by the Van Slyke analyses. At 29° C. and pH 7.53 the oxygen equilibrium curve has a half-saturation value of 4.5 mm. oxygen. Using this curve and the per cent saturations the partial pressures of oxygen in the blood samples can be obtained.

From the table it can be seen that in both pre-branchial and post-branchial blood samples, as a result of the unsaturated condition of the hemocyanin and the high oxygen affinity of this pigment, oxygen pressures are very low and practically all of the oxygen present is in combination with the hemocyanin. In each of the three cases about 0.75 ml. of oxygen was delivered to the tissues by 100 ml. of blood. The oxygen capacities of the whole bloods are higher than those reported for most other decapod crustacea and, correlated with this, samples of oxygenated blood were deep blue in color.

Blood for carbon dioxide analysis was taken from the bases of the legs of 5 specimens of *Cardisoma*. The 5 analyses yielded an average of 49.4 vol.% total carbon dioxide, the individual measurements being 46.0, 54.0, 46.8, 42.6 and 57.7 volumes per cent. These appear to be the greatest *in vivo* carbon dioxide concentrations reported for a decapod crustacean and are 2–5 times as large as figures given for other members of this group (Collip, 1920; Parsons and Parsons, 1923; Redmond, 1955; Wolvekamp and Waterman, 1960). Possibly this high concentration of carbon dioxide may be a result of aerial respiration plus a large alkaline reserve. The vast bulk of this carbon dioxide is presumably present as bicarbonate. The partition between dissolved gas and bicarbonate, however, cannot be established without first determining a carbon dioxide dissociation curve. This has not yet been done. Since *Cardisoma* is primarily aerial in respiration and the partial pressures of gases in the branchial chamber are unknown, it is not possible at present to make a reasonable estimate of the carbon dioxide tension in the blood.

Discussion

The oxygen equilibria data for the hemocyanin of *Cardisoma guanhumi* are qualitatively similar to those of the hemocyanins of other decapod crustacea. The sigmoid shape of the equilibrium curve and the presence of the normal Bohr effect are typical (Stedman and Stedman, 1925, 1926a, 1926b; Redfield, Coolidge and Hurd, 1926; Hogben and Pinhey, 1927; Redfield and Ingalls, 1933; Redfield, 1934; Redmond, 1955; etc.). Unusual, however, is the high oxygen affinity possessed by this pigment. Ordinarily, blood respiratory pigments having very low half-saturation pressures are associated with rather sluggish organisms or with ones living in areas where the environmental oxygen levels may be low (Prosser *et al.*, 1950). Neither of these situations appears to fit *Cardisoma*. Moving about on the surface of the ground, *Cardisoma* is nimble and its attitude is one of alertness. When disturbed it can move with considerable speed. Certainly, being an air breather, it need not experience low external oxygen pressures. The question then arises as to what, if any, is the significance of the high oxygen affinity of its hemocyanin.

One possibility is that it may be secondarily related to the aerial habits of this crab. One of the major problems facing *Cardisoma*, as a marine organism only partially adapted to a terrestrial life, must be the prevention of desiccation due primarily to water loss from the respiratory surfaces. Associated with this, most species of intertidal crabs which spend time out of water show a reduction in gill surface which is correlated with the per cent of time spent in air (Gray, 1957; Waterman, 1960). While other modifications also occur, such as vascularization and outgrowths of the branchial chamber lining to form new respiratory surfaces, adaptations of the branchial chamber which reduce water loss will almost necessarily also reduce the over-all rate at which oxygen will diffuse into the blood. Since the rate of diffusion is directly proportional to the partial pressure gradient, the farther to the left the oxygen equilibrium curve, the faster will oxygen penetrate. This, of course, results from the combination of the oxygen with the hemocyanin as the oxygen enters the blood, the internal partial pressure thus being kept very low. This maintenance of a high diffusion gradient may be the most important respiratory function of the blood respiratory pigments in many invertebrates, the increased oxygen-carrying capacity in such forms being perhaps of only secondary importance. The high oxygen affinity of *Cardisoma* hemocyanin may then represent a secondary adaptation compensating for the effect of water-conserving measures on oxygen uptake.

Another possibility, since practically nothing is known about the hemocyanins of tropical species, is that the low half-saturation pressure may be related to the relatively high environmental temperatures experienced by such forms. In this connection, it is interesting to note that a high oxygen affinity acts as a temperature buffer tending to stabilize loading pressures. As was previously mentioned, when the half-saturation pressures of the hemocyanins of a number of species are plotted against temperature on a semi-log graph, the resulting lines are roughly parallel. This means that absolute change in half-saturation per degree centigrade will be least in those blood respiratory pigments having the highest oxygen affinities. In the case of the hemocyanin of *Cardisoma* the changes are so small that, for environmental temperatures, the half-saturation pressure can almost be considered temperature-independent. However, without more data on the ecology of this organism, it is difficult to determine whether or not this feature is of physiological importance. The high oxygen affinity of *Procambarus* hemocyanin may similarly be an adaptation to fluctuating environmental temperatures and possibly oxygen concentrations.

The Van Slyke oxygen analyses of pre-branchial and post-branchial blood samples represent only three animals so that the average figures given in Table III may or may not reflect the population as a whole. In principle, however, the results agree completely with those of previous studies on three other species of decapod crustaceans, *Panulirus interruptus, Homarus americanus,* and *Loxorhynchus grandis* (Table II). In none of these species was the hemocyanin found to become saturated with oxygen as the blood passed through the gills. Consequently, the oxygen pressures in both pre-branchial and post-branchial blood samples were very low. Because of the high oxygen affinity of *Cardisoma* hemocyanin, post-branchial oxygen pressures (5–7 mm. Hg) were lowest in this species. The pre-branchial oxygen levels were very similar in all four species, being in the neighborhood of 2 to 4 mm. Hg.

About 0.75 volume of oxygen is delivered to the tissues by 100 volumes of

Cardisoma blood. This is a little more than that found for the other three forms but still indicates that only a small quantity of oxygen is delivered by a relatively large volume of blood. Since the hemocyanin of *Cardisoma* does not become saturated and the half-saturation pressure is very low, practically all of the oxygen carried by the blood is in combination with the hemocyanin. In practice, then, the hemocyanin of *Cardisoma* transports nearly all of the oxygen used by the tissues. Despite this finding there is still some question as to how essential is hemocyanin for oxygen transport in the decapod crustacea.

Zuckerkandl (1957a, 1957b) reported the very interesting finding that during certain stages of the molt cycle of the spider crab, *Maia squinado*, the hemocyanin concentration of the blood may drop to essentially zero, during which time oxygen physically dissolved in the blood supplies the animal's respiratory needs. The lowest levels occur during stages C_1 and C_2, with A_1, A_2, B_1, B_2, and C_3 also characteristically possessing very little hemocyanin. Associated with the low concentrations of hemocyanin, Zuckerkandl found the oxygen partial pressure of the post-branchial blood of these stages to vary from 50 to 120 mm. Hg. In later stages he reported that the hemocyanin content rose and that the post-branchial oxygen pressures were lower (40–50 mm.). These latter pressures, as Zuckerkandl states, are somewhat uncertain and may be too high. This uncertainty resulted from calculations based on an oxygen equilibrium curve published by Hogben (1926) which may not represent physiological conditions. The total quantity of oxygen found in the post-branchial blood of *Maia* (0.10–0.52 vol.%) is about that reported for *Loxorhynchus* but is lower than that found in *Panulirus* and *Cardisoma*. It may or may not be significant that *Maia* and *Loxorhynchus* are both spider crabs and are rather sluggish as compared with the other two species.

Unlike Zuckerkandl, the author does not believe that the much higher post-branchial oxygen tensions reported for *Maia* indicate an important physiological difference for this species from those in which the post-branchial oxygen tensions are low. The high oxygen partial pressures in *Maia* blood are a direct consequence of the absence or low concentration of hemocyanin and do not necessarily indicate the oxygen tensions at which the tissues must operate. In the absence of a respiratory pigment, if the blood is to carry any appreciable quantity of oxygen away from the gills, it must carry it at a high partial pressure. Measurements of pre-branchial oxygen partial pressures in *Maia* would be of interest. It is difficult to assess the relative importance in blood of high oxygen tensions versus larger quantities of oxygen at lower pressures but it would seem that, so long as the tissues are capable of normal respiration at low pressures, the quantity rather than the tension would be of more importance. The intimate relationship between the blood and tissues would minimize any difficulties in the transfer of oxygen that might be expected to result from a small diffusion gradient between tissues and blood.

Why the hemocyanin should tend to disappear immediately following molt is not known. Zuckerkandl suggests that it may serve as a protein store. The period of most active tissue growth, stages C_1 and C_2, coincides with the period during which the hemocyanin is least concentrated. Possibly this protein serves as a source of amino acids for the rapid growth that occurs at this time. That the hemocyanin can serve as a food source seems likely since this substance considerably decreases in amount during starvation (unpublished observations on *Uca pugilator* and *Cardisoma guanhumi*). It would appear that during the period of presumably greatest gill permeability, when the hemocyanin would be least necessary as a respiratory pigment, this substance is used for another purpose. The primary question to be answered, however, is whether the findings for *Maia* are typical of the decapod Crustacea. In particular, it would be of interest to follow the hemocyanin level of the blood during the molt cycle of a more active species or one in which the hemocyanin concentration is characteristically high.

The hemocyanin in the blood of the decapod Crustacea may serve several functions. As a circulating protein, it is an important buffer and is largely responsible for the colloid osmotic pressure of the blood. Zuckerkandl's findings suggest that there may be other unknown functions. The very special properties of this substance, together with its location in the blood, however, make it difficult not to believe that its principal role is that of a respiratory pigment, whether it acts primarily as an oxygen transporter, to increase the oxygen capacity of the blood, or to speed the diffusion of oxygen. If this is not so, it seems highly improbable that its peculiar ability to combine reversibly with oxygen in appropriate pressure relationships would have evolved, or, having once developed, been perpetuated in such a large group of animals. This is not to say that it is necessarily essential for respiration. Probably all organisms may suffer the loss of various structures and continue to live although these structures normally play a role in the life of the organism and confer upon it certain advantages. The respiratory advantage conferred by hemocyanin consists of a greater supply of oxygen delivered to the tissues for a given quantity of circulatory work than would be delivered in its absence. Some of this extra oxygen may be used regularly; *Cardisoma* extracts more oxygen from a given volume of blood than could be carried in solution; or it may serve as a reserve which can be drawn upon during periods of greater activity or other stress.

I am deeply indebted to Professor D. M. Steven of the Zoology Department of the University College of the West Indies for providing me with space and facilities in his Department, and to the other members of the staff for their hospitality and help. In particular I wish to thank Dr. Ivan Goodbody for his assistance in collecting and many other matters which greatly aided me in this investigation. My thanks are due also to Dr. Charles Gifford, of the University of Miami Marine Laboratory, for his help in collecting the Florida specimens of *Cardisoma*.

SUMMARY

1. The hemocyanin of the land crab, *Cardisoma guanhumi,* under physiological conditions, possesses a high oxygen affinity.

2. The oxygen equilibrium curve is sigmoid, with a fairly high degree of interaction occurring between the oxygen-combining sites of the hemocyanin molecule.

3. As in all other crustacean hemocyanins investigated, a normal Bohr effect is present.

4. Van Slyke analyses of the oxygen content of pre-branchial and post-branchial blood samples indicated that nearly all of the oxygen present was in combination

with the hemocyanin; consequently the oxygen partial pressures throughout the circulatory system were very low. The hemocyanin did not become saturated with oxygen during passage through the gills. These results are similar to those previously found for three other species of decapod crustaceans.

5. The carbon dioxide content of the blood was very high, averaging almost 50 volumes per cent.

6. It is shown that the low half-saturation value of the hemocyanin confers a stability of loading pressure in the face of changing environmental temperature. It is speculated that the high oxygen affinity may also represent an adaptation tending to counter the effect of water-conserving measures on the rate at which oxygen diffuses through the respiratory surfaces into the blood.

7. Zuckerkandl's studies on the crab, *Maia squinado,* are discussed and it is tentatively concluded that, though the hemocyanin of the decapod Crustacea may serve several functions and may not always be essential for respiration, its primary function is that of a blood respiratory pigment.

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ORGANIZATION AND COMPOSITION OF THE AMPHIBIAN YOLK PLATELET. I. INVESTIGATIONS ON THE ORGANIZATION OF THE PLATELET[1]

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The importance of yolk platelets in the embryonic and larval development of amphibians is indicated by the large proportion of oocytic material contained in these dense, subcellular structures. Of the total egg nitrogen, 69–72% is found in the yolk platelet fraction (Gregg and Ballentine, 1946; Lawrence, Miall, Needham and Shen, 1944). But the precise roles of yolk platelet lipo- and phosphoproteins are not yet adequately defined, nor are the mechanisms of yolk synthesis and yolk utilization well understood. A review of the present state of knowledge concerning the development, composition, organization and utilization of the yolk platelet has been presented by Ringle (1960).

A broad appreciation of the factors involved in yolk synthesis and utilization requires an adequate knowledge of the organization and composition of the yolk platelet. A number of investigations have been performed on amphibian yolk platelets *in ovo* as well as *ex ovo*, including the following: chemical analyses by McClendon (1909), Fauré-Fremiet and de Streel (1921), Panijel (1950), Schjeide, Levi and Flickinger (1955), and Gross and Gilbert (1956)—a review of many of the early investigations is given by Needham (1931); cytochemistry by Voss (1927), Hibbard (1928), Holtfreter (1946a) and Laufer (1949)—a review of some of the cytochemical investigations on amphibian eggs is given by Brachet (1950); electron microscopy by Kemp (1956), Eakin and Lehmann (1957), Wischnitzer (1957), Karasaki and Komoda (1958) and Ward (1959); immunological analyses by Cooper (1946, 1948, 1950), Flickinger and Rounds (1956), and Glass (1959); electrophoresis by Flickinger and Nace (1952), Barth and Barth (1954), and Schjeide *et al.* (1955); ultracentrifugation by Schjeide *et al.* (1955), Gross and Gilbert (1956) and Flickinger and Schjeide (1957); viscosity and flow-birefringence studies by Lawrence *et al.* (1944); and enzymology by Harris (1946), Brachet (1950), Panijel (1950), Recknagel (1950), Gross (1954) and Nass (1956). These studies have contributed much towards elucidating the organization and composition of the yolk platelet, but there are still many important aspects of the problem left undecided or uninvestigated.

Since yolk platelets are readily isolated from ovarian eggs, as by the homogenization-centrifugation method of Essner (1954), investigations on platelets rela-

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tively free from other material are feasible. Some of the previously mentioned studies were performed on such washed, isolated platelets. Analyses on whole, washed platelet suspensions offer a means of determining the composition of amphibian yolk. By lysis with salts the yolk can be fractionated into soluble (YP) and insoluble ("ghost" material) fractions. In addition, washed platelet suspensions provide a readily available source of platelets for other types of investigation (*e.g.,* micromanipulation). One question of importance, however, concerns the composition of the washed platelet suspension, particularly the "ghost" fraction which remains after lysis. If this "ghost" fraction is to be analyzed as a yolk platelet fraction, it must be shown to be largely yolk platelet in origin.

The studies to be reported here are primarily concerned with the following:

a. The nature of the "ghost" material.
b. The nature of the surface of the platelet.
c. The behavior of the platelet components during lysis by sodium and calcium ions.
d. The organization of the yolk components within the platelet.

Materials and Methods

1. *Preparation of washed yolk platelets*

Washed yolk platelets were prepared from homogenized ovarian eggs of *Rana pipiens* by the differential centrifugation method of Essner (1954). All homogenizations and centrifugations were carried out using cold solutions (refrigerator or ice bath temperatures) and chilled tubes. Buffers used were either pH 7.0 phosphate buffer (0.05 *M* KH_2PO_4-NaOH) or pH 7.2 Tris (hydroxymethyl) aminomethane-HCl 0.05 *M* buffer. Ovaries were dissected from freshly-pithed frogs, washed in several changes of buffer, and homogenized by hand with a Potter-Elvehjem homogenizer containing a small amount of buffer. The homogenate was filtered through a double thickness of cheesecloth and the filtrate diluted with 100 ml. of additional buffer for the material from each group of 2–3 frogs. The platelets were suspended and washed in 4–5 changes of buffer and in 4–5 changes of 0.1 *M* NaCl. Centrifugations were conducted for 4–6 minutes each at 140–150 × *g*. Washed platelet preparations were either used immediately or were refrigerated at 3–5° C. until used.

2. *Micromanipulation*

Micromanipulation experiments were performed using a Carl Zeiss (Jena) sliding micromanipulator. Microneedles and micropipettes were drawn by hand from 0.7–0.8-mm. external diameter glass tubing with the aid of a gas microburner. The techniques used were essentially those described by Chambers and Kopac (1950).

3. *Lysis in coverslip-slide diffusion chambers*

Simple chambers were constructed by affixing No. 1 thickness coverslips to glass slides by means of a thin layer of petroleum jelly applied to three edges of the coverslip. A drop of platelet suspension was placed on the coverslip and the slide then gently pressed down on the coverslip, resulting in a platelet-containing chamber open at one end. After 10–15 minutes the slide was inverted (*i.e.*, the coverslip-bearing surface placed uppermost) and the platelets adhering to the coverslip observed with a microscope. Application of a drop of saline (1.0–2.0 *M* NaCl or $CaCl_2$) to the open end of the chamber allowed saline to diffuse into the chamber at a rate sufficiently slow to permit the observation of lysis of individual platelets as the saline front passed.

4. *Histological and cytochemical investigations*

a. Fresh preparations

Washed yolk platelets and "ghost" material (the insoluble residue of lysed platelet preparations) were stained in 0.05% toluidine blue O (C.I. 925) made up in 0.05 *M*, pH 7.0 phosphate buffer plus 0.1 *M* NaCl (1 : 1 by volume).

b. Fixed preparations

Pieces of *Rana pipiens* ovary were fixed for 24 hours in Smith's modification of Tellyesnicky's bichromate mixture, washed, and imbedded in paraffin (Tissuemat 56–58.5° C. m.p.) following the method described by Laufer (1949). "Ghost" material was fixed for 5 hours in Bouin's picrol-formol solution (Guyer, 1936) and washed in changes of 50 and 70% ethanol. It was then carried through the usual higher concentrations of ethanol, ethanol + xylol, and xylol. Imbedding was done in Tissuemat as for ovarian tissue. Paraffin sections were cut 7 μ thick and affixed to slides with Mayer's albumin.

Feulgen nucleal reaction. Slides of sectioned ovary and "ghost" material were stained by the Feulgen method as described by Lillie (1954). Schiff's leucofuchsin was prepared with basic fuchsin (C.I. 677) by the traditional method.

Periodic acid-Schiff (PAS). The method employed was the short PAS variant described by Lillie (1954).

Heidenhain's iron hematoxylin. A conventional method (Gatenby and Beams, 1950) was used with minor modifications. After staining in 0.5% aged iron hematoxylin, sections were destained in 2% ferric alum, washed for one hour in running tap water and counterstained in 0.5% eosin-Y. Some slides previously PAS-treated were also stained in hematoxylin, without counterstaining in eosin.

Basic fuchsin and acid fuchsin. Slides were stained for one hour in 0.5% aqueous basic fuchsin (C.I. 677) or acid fuchsin and then briefly washed in distilled water.

5. *Electron microscopy of yolk platelets and YC-fragments*

Washed yolk platelets were prepared from the homogenized ovarian eggs of three frogs (*Rana pipens*) by the homogenization-centrifugation procedure. A portion of this washed platelet suspension was lysed in 0.5 *M* NaCl and centrifuged to yield a clear, "ghost"-free yolk solution (YP_{na}). Platelet-like YC-fragments were prepared from this YP_{na} by a reduction in salinity, as described elsewhere (Ringle, 1960; Ringle and Gross, 1962). In brief, the viscous, dense YC precipitate, which forms following the reduction of YP_{na} salinity to 0.30 *M* NaCl, was made brittle by transferring it to 0.1 *M* NaCl. After the hardened YC material was broken up by homogenization in a Potter-Elvehjem homogenizer, the resulting YC-fragments were washed in four changes of 0.1 *M* NaCl by centrifugation.

Portions of washed yolk platelet suspension and YC-fragment suspension were separately fixed and imbedded. All fixing, washing and infiltrating procedures were carried out in 12-ml. conical centrifuge tubes with low-speed centrifugation to facilitate separation of the fixed yolk materials from their solutions. The fixative used was buffered (pH 7.2–7.4) 1% osmium tetroxide prepared to an osmolarity of 0.14 by the method of Gross, Philpott and Nass (1958). One-half-milliliter quantities of yolk material were added to 8 ml. of fixative and fixed for 5 minutes. The fixed yolk materials were run up through changes of alcohol (30, 50, 70, 83, 95% and absolute alcohol) and then transferred to a 1:1 mixture (by volume) of absolute ethanol and *n*-butyl methacrylate-methyl methacrylate (4:1). After two one-hour changes of the 4:1 methacrylate mixture the fixed materials were suspended in 4:1 methacrylate containing 2% Luperco catalyst and transferred to gelatin capsules which had been previously half-filled with 4:1 methacrylate and pre-polymerized for four hours. The capsules were placed in a drying oven at 45° C. for three days and then exposed to ultraviolet light for 5 hours.

Sections were cut using a microtome described by Philpott (1955) and spread out on an acetone-water mixture. The sections were supported on a Formvar-100 mesh copper grid. Examination was done with a RCA model EMU-2C electron microscope (250 μ condenser aperture and 65 μ objective). The microscope was tested for and found free of astigmatism previous to examination of the yolk materials.

Results

1. *Experiments on isolated platelets and YC-fragments*

a. Micromanipulation

The responses of yolk platelets and YC-fragments to microdissection were similar in similar media. In distilled water or 0.1 *M* NaCl these yolk materials were brittle and readily dissected or fragmented by glass microneedles (Figs. 1, 2, 3). This brittleness was found not only for washed platelets and YC-fragments but also for unwashed platelets freshly extruded from crushed eggs. Like the platelets of *Rana pipiens*, platelets of *Rana catesbiana* were brittle at low salt concentrations. Occasionally, fragments of split platelets would remain feebly attached at one margin, thus indicating the presence of a pliable surface coat. This "coat" was more apparent in stored platelets and in platelets briefly exposed to 0.28–0.30 *M* NaCl prior to dissection.

Platelet brittleness was also evident in simple experiments involving the crushing of yolk platelets beneath a coverslip by gentle pressure applied to the coverslip with a steel dissecting needle or pencil tip. Directly beneath the applied pressure platelets were flattened out into a thin sheet, the margins of which resembled pseudopodia. The gentler pressure at the periphery of the coverslip, however, merely fragmented the platelets.

In 0.28–0.30 *M* NaCl YC-fragments and yolk platelets were soft and pliable. They became more or less spherical and were readily deformed by the microneedles, although the tendency to become globular was much reduced for platelets not freshly prepared. If these softened platelets were returned to a lower salt

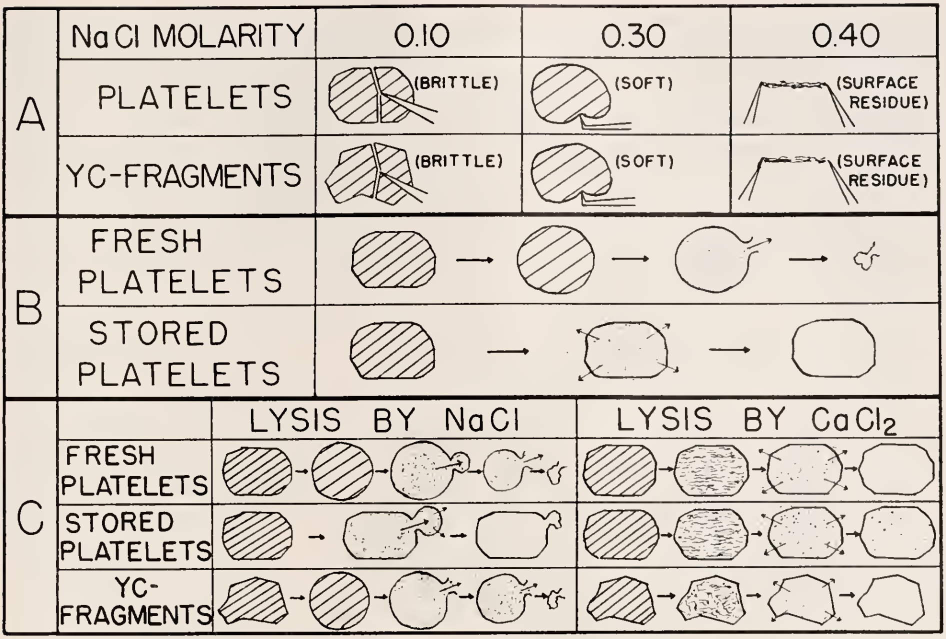

A
NaCl MOLARITY
0.10
0.30
0.40
PLATELETS
(BRITTLE)
(SOFT)
(SURFACE RESIDUE)
YC-FRAGMENTS
(BRITTLE)
(SOFT)
(SURFACE RESIDUE)
B
FRESH PLATELETS
STORED PLATELETS
C
LYSIS BY NaCl
LYSIS BY CaCl2
FRESH PLATELETS
STORED PLATELETS
YC-FRAGMENTS

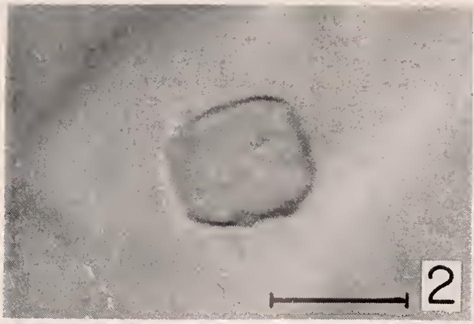
FIGURE 1. Responses of isolated yolk platelets and YC-fragments to micromanipulation and lytic salt solutions. A: micromanipulation with glass microneedles. B: lysis by NaCl solutions directed by micropipettes. C: lysis in coverslip-slide diffusion chambers.

concentration (below 0.28 *M* NaCl), they again became brittle (Figs. 4, 5). At NaCl concentrations above 0.30 *M* most of the yolk solubilized, leaving behind an insoluble surface coat which could be stretched or manipulated by microneedles.

By directing a flow of NaCl or $CaCl_2$ solutions with micropipettes at platelets in distilled water or 0.1 *M* NaCl, it was possible to observe the lysis of individual platelets and to influence the extent of their lysis. A continuous flow of 1.0–2.0 *M* NaCl resulted in a rapid lysis of all the platelets in the saline path. Freshly prepared, washed platelets became spherical and rapidly burst. Platelets from preparations which had been stored for several days, however, usually did not round up and, after lysis, left an insoluble residue approximately the size and shape of the platelet before lysis. If the NaCl jet was applied tangentially, a partial lysis of the platelet sometimes occurred, leaving a portion of the platelet intact.

Platelet lysis also resulted from jets of 0.1–1.0 *M* $CaCl_2$, although with this salt lysis was only occasionally preceded by a rounding of the platelet prior to the escape of solubilized yolk. If the flow of $CaCl_2$ was terminated prior to the escape of yolk, the platelet could be fragmented by microneedles. Fragments from these dissected platelets tended to remain attached to a pliable surface coat; and, when the flow of $CaCl_2$ was resumed, the fragments lysed, leaving behind a faintly visible surface coat.

The results of these micromanipulation studies, as well as the following lysis investigations, are summarized in Figure 1.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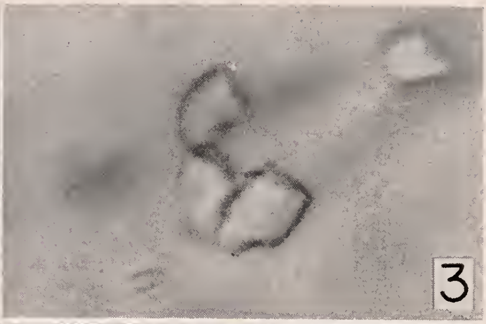
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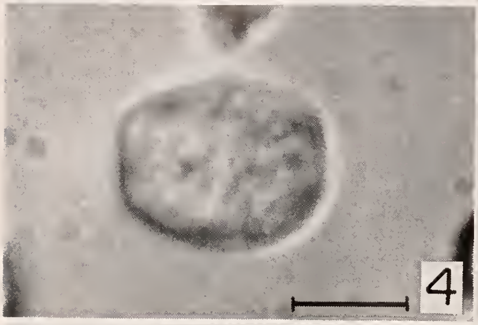
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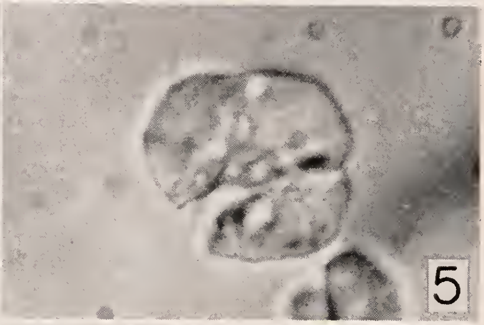
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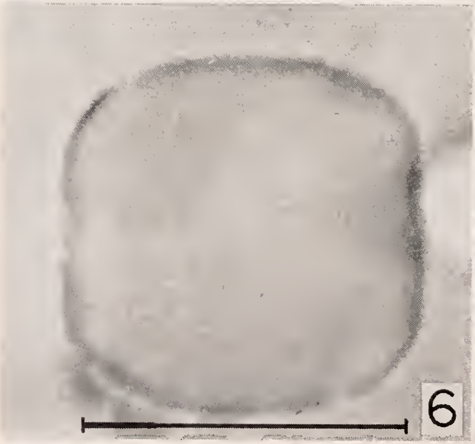
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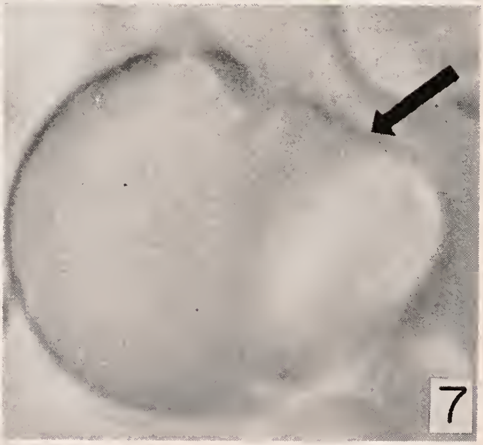
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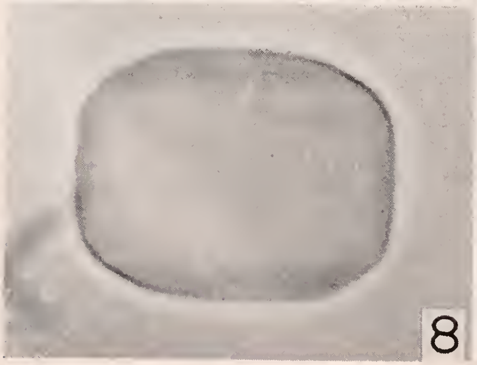
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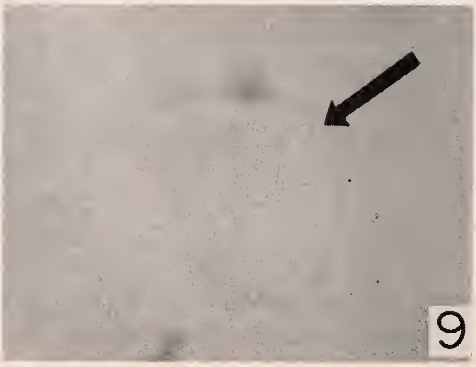
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b. Lysis in coverslip-slide diffusion chambers

Lysis of freshly washed platelets. Lysis by NaCl was usually accompanied by an initial rounding and slight swelling of the platelet as the saline front passed, although some platelets did not round up prior to lysis. The apparent swelling may have been caused by a slight flattening of the yolk mass, since these preparations were viewed from above. Within a second or two after rounding one or more blebs appeared on the platelet surface, followed by a shrinking of the platelet surface as the bleb or blebs enlarged. Finally solubilized yolk escaped from the blebs, leaving behind a shrunken ghost of the lysed platelet (Figs. 1, 6, 7).

$CaCl_2$ did not ordinarily cause a marked swelling or rounding of platelets, although a few did round up prior to lysis. Lysis by $CaCl_2$ typically involved a marked wrinkling of the platelet interior, with the direction of wrinkling parallel to the long axis of the platelet. Then solubilized yolk appeared to escape from the entire surface of the platelet, not from one or several localized ruptures as in the case of lysis by NaCl. The residual surface coat or ghost retained approximately the size and shape of the platelet before lysis and did not show the marked shrinking which occurred after lysis by NaCl, although some shrinkage was common (Figs. 8, 9).

Lysis of unwashed platelets. Platelets were prepared by crushing ovarian eggs in a small quantity of buffer, about 0.05–0.1 ml. per egg (either 0.05 *M*, pH 7.2 Tris or pH 7.0 phosphate buffer). Most of these unwashed platelets did not lyse readily with either NaCl or $CaCl_2$ in the diffusion chamber. When lysis did occur it was both delayed and sporadic. Within a minute or two after the passage of NaCl some of the platelets began to show striae or wrinkles parallel to their long axes, while others rounded up slightly. These changes were followed by vacuole formation within the platelet and by an irregular swelling of the platelet. Those platelets which did lyse left behind a shrunken ghost, although many platelets were still intact or swollen 5 minutes or longer after passage of the saline front (Figs. 10, 11, 12). Prolonged exposure (5–10 minutes) to $CaCl_2$ caused a marked swelling of many platelets, but lysis was relatively incomplete.

After dilution of these unwashed platelet suspensions with 2–3 volumes of buffer, lysis by saline was still abnormal when compared with the lysis of washed platelets. Lysis was delayed for 10–30 seconds or more after passage of the saline front, and it occurred at random among the platelet population without detectable rounding or bleb formation. Lysis in the presence of egg cytoplasm

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FIGURE 2. Washed yolk platelet in distilled water. Scale marker indicates 10 μ for Figures 2 and 3.

FIGURE 3. Same platelet as in Figure 2 following dissection. Tip of micropipette visible at upper right.

FIGURE 4. Washed yolk platelet (pretreated in 0.3 *M* NaCl) in 0.2 *M* NaCl. Scale marker indicates 10 μ for Figures 4 and 5.

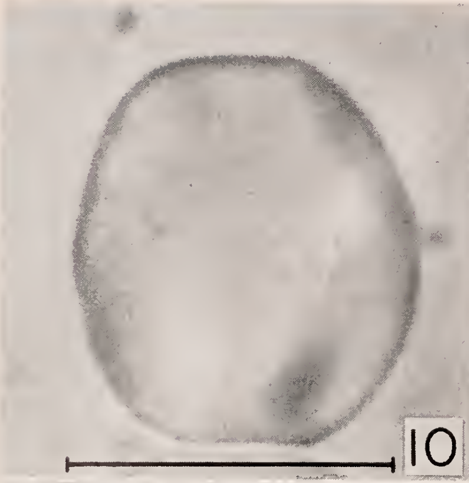
FIGURE 5. Same platelet as in Figure 4 following dissection.

FIGURE 6. Washed yolk platelet in 0.1 *M* NaCl in coverslip-slide diffusion chamber. Scale marker indicates 10 μ for Figures 6–9.

FIGURE 7. Same platelet as in Figure 6 during lysis by NaCl. Note shrunken portion of platelet at right (arrow) and large bleb at left.

FIGURE 8. Washed yolk platelet in 0.1 *M* NaCl in coverslip-slide diffusion chamber.

FIGURE 9. Ghost (arrow) of platelet shown in Figure 8 after lysis by $CaCl_2$.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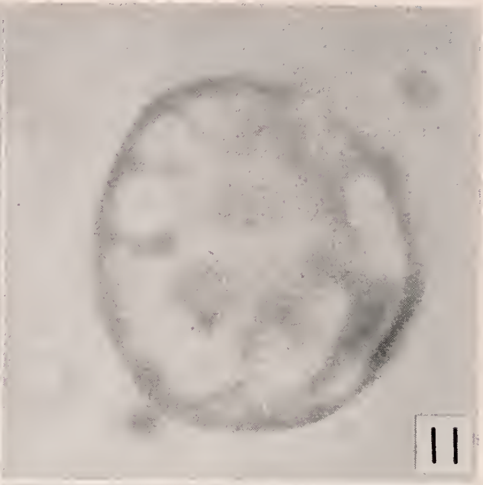
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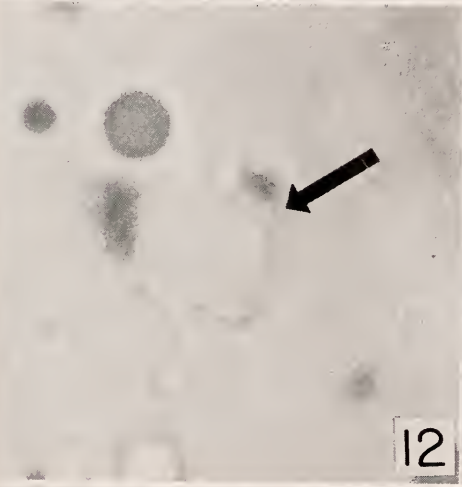
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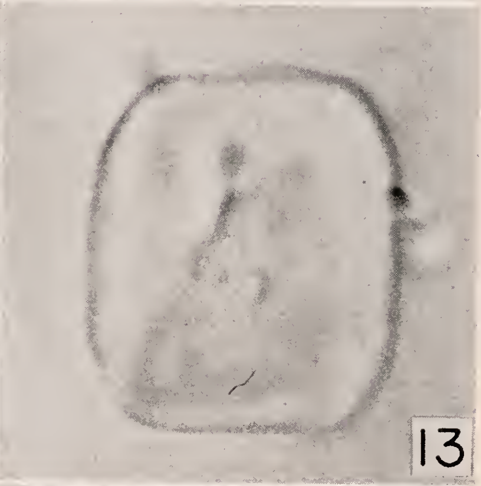
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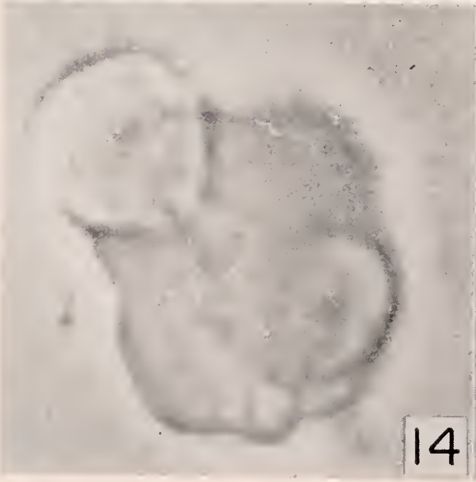
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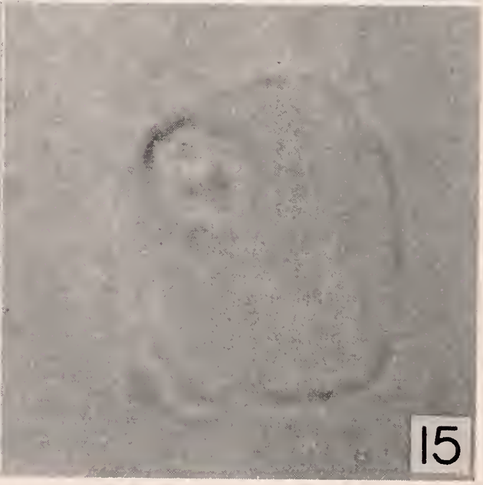
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resulted in the appearance of numerous lipid droplets, which occasionally clustered around the residual ghosts of lysed platelets. Platelets from these crushed egg suspensions, if washed in several changes of buffer, reacted to lytic salt solutions like platelets from the homogenization-centrifugation procedure. Thus, relatively long exposure (one hour or more) to the cytoplasmic materials of crushed eggs did not permanently alter the lytic properties of platelets.

Lysis of stored platelets. NaCl-lysis of washed platelets which had been stored under refrigeration (3–5° C.) for 6–10 days did not usually produce a rounding or swelling of the platelets. Instead, bleb formation occurred at one of several places on the platelet surface, and particles in Brownian motion streamed from the platelet interior into the swelling blebs. After a few seconds fluid yolk escaped from the blebs, leaving behind an insoluble ghost which retained the approximate size and shape of the original platelet. Shriveled residues of the surface blebs were also visible (Figs. 1, 13, 14, 15). During lysis by $CaCl_2$ the interior of these stored platelets became wrinkled in appearance, as was also true for freshly prepared platelets. After lysis by $CaCl_2$ particles exhibiting Brownian motion were trapped within the ghost and could be seen as long as 15 minutes after lysis (when observations were terminated).

Lysis of YC-fragments. Lysis of YC-fragments by NaCl proceeded initially as for freshly washed platelets by rounding up, but bleb formation was not observed. Instead, the spherical yolk mass swelled and finally burst, leaving behind a shrunken, sac-like residue. YC ghosts did not readily remain attached to the coverslip but were usually swept away by the diffusing saline. Lysis of YC-fragments by $CaCl_2$ proceeded as for freshly washed platelets.

2. *Histological and cytochemical investigations*

a. Fresh preparations

Washed yolk platelets of *Rana pipiens* stained metachromatically in buffered toluidine blue O, the platelet surface staining darker than the platelet interior. "Ghost" material stained only lightly, except for scattered patches (apparently cell nuclei, insoluble surface residues of lysed platelets, and unidentified granules). Some surface residues of lysed platelets enmeshed in "ghost" substance retained the size and shape of unlysed platelets. Preparations of "ghost" material from platelet suspensions of *Rana catesbiana* also contained larger (40 μ or more in diameter) amorphous metachromatic patches.

FIGURE 10. Yolk platelet from crushed egg in cytoplasm, slightly diluted with pH 7.0 phosphate buffer in coverslip-slide diffusion chamber. Scale marker indicates 10 μ for Figures 10–15.

FIGURE 11. Same platelet as in Figure 10 after passage of diffusing NaCl front. Note vacuoles within the platelet.

FIGURE 12. Ghost (arrow) of platelet shown in Figures 10–11. Note lipid droplets.

FIGURE 13. Washed, stored (refrigerated 10 days) yolk platelet in 0.1 M NaCl in coverslip-slide diffusion chamber.

FIGURE 14. Same platelet as in Figure 13 during lysis by diffusing NaCl. Note several blebs formed at surface of platelet.

FIGURE 15. Ghost of platelet shown in Figures 13–14 with surface bleb residue.

b. Fixed preparations

The results of the following staining procedures on fixed ovary and "ghost" material are summarized in Table I.

Feulgen nuclear reaction. Yolk platelets of sectioned oocytes showed a faint fuchsin color in acid-hydrolyzed slides, whereas control slides gave either a negative or much reduced reaction. Extruded platelets (*i.e.,* platelets released from eggs cut during dissection of the ovary and fixed along with intact ovarian eggs) were stained slightly or not at all when compared with platelets fixed *in ovo.*

"Ghost" material contained a large number of distorted, Feulgen-positive patches, resembling the nuclei of follicle cell membranes in size and distribution.

Periodic acid-Schiff (PAS). Sectioned oocytes exhibited a large amount of PAS-positive granular material distributed among the platelets and adhering to platelet surfaces. Platelets *in ovo* showed distinctly PAS-positive margins, but only faintly positive or PAS-negative interiors. Extruded platelets, however, were either PAS-negative or showed only slightly stained margins (Figs. 16, 17). Follicle membranes were markedly PAS-positive and in control slides stained faintly, which was similar to the staining behavior of the bulk of the "ghost" material.

Heidenhain's iron hematoxylin. Sectioned ovary counterstained with eosin showed intensely eosinophilic follicular membranes containing hematoxylin-stained nuclei. Within eggs the margins of yolk platelets were darkly stained by hematoxylin, although platelet interiors were either unstained or only lightly tinted. Extruded platelets showed less intensely stained surfaces than did platelets *in ovo.* The matrix of "ghost" material, like the follicular membranes, was eosinophilic and contained a number of hematoxylin-stained nuclei.

Oocyte sections first stained by the PAS-method and then stained with hematoxylin were much more markedly stained than sections not previously PAS-

Table I

Results of histological and cytochemical staining procedures

Staining procedure	"Ghost" material		Ovary				
	Nuclei	Matrix	Follicle cells	Platelets *in ovo*		Extruded platelets	
				Surface	Interior	Surface	Interior
Feulgen nuclear	++++	±*	++++** ±*†	+	+	±	±
Periodic acid-Schiff		+++	+++	++++	±	±	−
Hematoxylin + eosin	H++++	E++ H++††	E+++ H+++**	H+++	H−	H+	H−
PAS + hematoxylin		H+++††		H++++	H±	H++	H±
Basic fuchsin	+++	+	+† +++**	+++	±	+	±
Acid fuchsin		++++	++++	++	−	±	−

* For both experimental and control sections.
** Nuclei.
† Non-nuclear matrix.
†† Small scattered patches.

treated. Platelets *in ovo* showed more darkly stained surfaces as well as some stain in the platelet interiors. Extruded platelets were also stained, but less than platelets within oocytes. The ground substance of the follicular membranes did not stain with hematoxylin, nor did the bulk of the sectioned "ghost" material (which did, however, contain some hematoxylin-stained patches resembling surface residues of lysed platelets).

Basic fuchsin and acid fuchsin. The interiors of platelets in sectioned oocytes were moderately to weakly stained by basic fuchsin, whereas their surfaces were darkly stained. Platelets did not stain as intensely in acid fuchsin, although they did possess a distinctly fuchsin-colored surface. The fine granular material distributed among the platelets stained much more deeply with acid fuchsin than with basic fuchsin. As for the other staining reactions previously described, extruded platelets did not stain as intensely as platelets *in ovo,* except for platelets surrounded by extruded cytoplasmic material. Follicle membranes stained lightly in basic fuchsin (except for their darkly stained nuclei) but were intensely colored by acid fuchsin. "Ghost" material stained like follicle membranes with these dyes.

3. *Electron microscopy of platelets and YC-fragments*

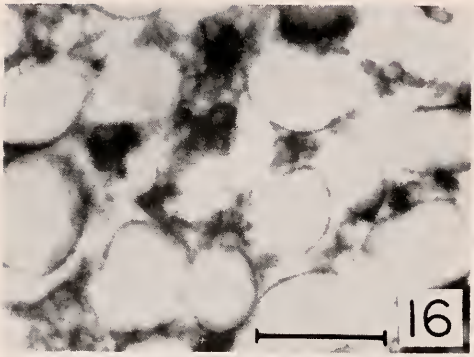
Electron microscopy of osmium-fixed, washed yolk platelets of *Rana pipiens* revealed an orderly arrangement of yolk components within the platelet (Figs. 18, 19). Approximately 36-Å wide lamellae (or bands of closely packed units) were oriented more or less parallel to the long axis of the platelet. Periodicity, the distance between centers of adjacent lamellae, was 72 Å. Peripheral electron-dense material sometimes gave the appearance of an irregular outer coat, but examination showed this surface material to be only a more opaque continuation of the internal yolk (since periodic lamellae continued into this "coat").

The organization of yolk components in YC-fragments was not as distinctly lamellar as that of platelets, although micrographs of the YC-fragments showed a compact structure as well as some indication of periodicity (Fig. 20). There was an irregular banding by linearly arranged units similar in size and appearance to the electron-dense substance comprising the lamellae of yolk platelets.

Discussion

1. *Experiments on isolated platelets and YC-fragments*

Washed yolk platelets and YC-fragments were rigid, brittle bodies in calcium-free, low-saline solutions (less than 0.28 *M* NaCl). This rigidity would be expected from the normally angular, crystal-like shapes of yolk platelets *in ovo.* Observations by Holtfreter (1946b) on the breakup of platelets during intracellular digestion also indicated a rigid nature for these bodies. However, Holtfreter (1946a) claimed a plastic consistency for amphibian yolk platelets, since he found that platelets squeezed between glass plates spread out into lobular sheets. This plasticity under heavy pressure applied to the entire surface of the platelet was confirmed in the studies reported here. Nevertheless, locally applied pressure with a microneedle tip revealed that platelets are sufficiently brittle to be readily split into fragments. Since YC-fragments showed the same behavior during micro-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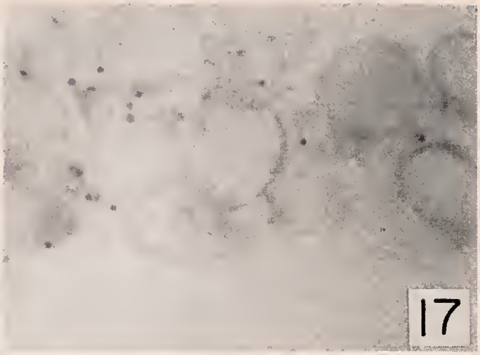
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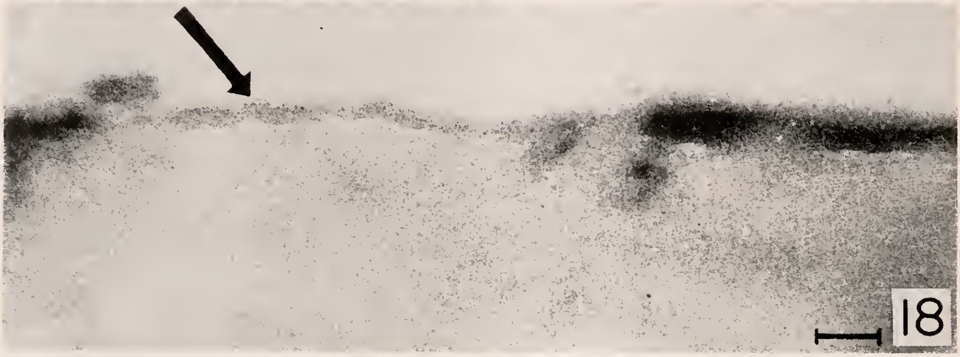
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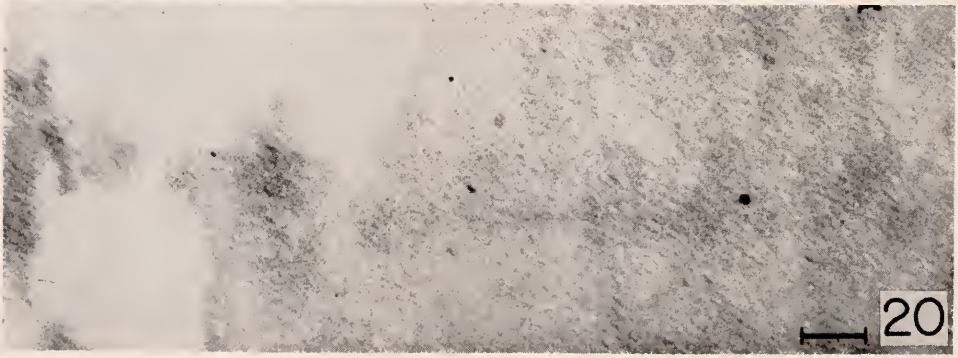
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manipulation, this suggests a similar packing of the yolk constituents in platelets and YC-fragments.

The solubilizing effects of salts on amphibian yolk are well known. As early as 1921 Fauré-Fremiet and de Streel showed that 10% NaCl (as well as strongly acid or alkaline solutions) dissolved the yolk platelets of *Rana temporaria*. Needham (1931) discussed the solubility of many kinds of yolk in salt solutions. Other investigations on the effects of ions on yolk solubility have been reported by Holtfreter (1946a), Terry (1950), Essner (1954), Gross (1954), Flickinger (1956), and Flickinger and Schjeide (1957). Yolk platelets are readily solubilized by 0.4 M concentrations of monovalent cation salts, and the soluble yolk is readily precipitated by dilution (to less than 0.28 M in the case of NaCl). Solubilization by salts of divalent cations occurs at much lower concentrations (*e.g.*, 0.003 M $CaCl_2$) and prolonged exposure to lytic concentrations of calcium renders yolk permanently soluble (Gross, 1954; Flickinger, 1956). Such differences in the response of amphibian yolk to NaCl and $CaCl_2$ point to different mechanisms for the solubilization of yolk by monovalent and divalent cations. Observations reported here on individual platelets during lysis likewise indicate differences in the effects of NaCl and $CaCl_2$ (degree of platelet swelling, bleb formation, escape of fluid yolk, wrinkling, etc.).

The effect of near-lytic concentrations of NaCl (0.28–0.30 M) was a marked increase in platelet plasticity. Although there were still sufficient binding forces to prevent yolk components from going into solution, platelets readily lost their angularity and could be easily deformed by microneedles. The plastic consistency of platelets in 0.28–0.30 M NaCl was similar to that of yolk precipitated from solution by a reduction of NaCl concentration to the same molarity (YC). As reported elsewhere (Ringle and Gross, 1962) the volume occupied by this plastic YC material is equal to the volume of the packed platelets before lysis, again indicating the platelet-like organization of yolk components in YC.

Micromanipulation and observations of washed platelets during lysis showed that isolated platelets possess an insoluble surface different from the soluble material within the platelet. However, whether or not this surface material exists as such *in ovo* is questionable. Since the surface coat increased in prominence with time after isolation of platelets from the egg, one suspects that even the coats of freshly washed or extruded platelets may be preparation artifacts resulting from a surface denaturation of yolk components. Also, surface coats were found on the platelet-like YC-fragments prepared from solubilized yolk. Hence, one must reserve judgment concerning the presence or absence of surface coats on platelets

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FIGURE 16. PAS-stained yolk platelets *in ovo*. Note staining of platelet surface. Scale marker indicates 10 μ for Figures 16–17.

FIGURE 17. PAS-stained extruded yolk platelets. Note platelet surfaces are less intensely stained than in Figure 16.

FIGURE 18. Electron micrograph of a portion of an osmium-fixed washed yolk platelet. Note orientation of yolk components in bands approximately parallel to the platelet surface. Electron-dense surface (arrow) also shows periodic banding. Scale marker indicates 0.1 μ.

FIGURE 19. Electron micrograph of platelet showing distinct periodicity (72 Å). Scale marker indicates 0.1 μ.

FIGURE 20. Electron micrograph of sectioned YC-fragment showing an irregular banding of yolk components. Scale marker indicates 0.1 μ.

in ovo from evidence involving isolated platelets. The presence of a coat on isolated platelets only indicates the possibility that such a coat normally exists *in ovo*.

2. *Histological and cytochemical investigations*

a. Nature of the "ghost" material

Washed yolk platelet suspensions in the presence of lytic concentrations of salts yielded soluble yolk and a small insoluble residue referred to here as "ghost" material. This "ghost" material was investigated by several histological and cytochemical methods to establish whether or not it was largely made up of the insoluble surface residues of lysed platelets. The results of staining reactions for both fresh and fixed material strongly suggest that the bulk of the "ghost" material consists of fragmented ovarian membranes plus only a small quantity of lysed platelet ghosts. The large metachromatic patches found in "ghost" material of *Rana catesbiana* may have been fragments of the metachromatic egg jelly layer, since this bullfrog material was prepared relatively late in the season (May 15th). Kelly (1954) has proposed that the inner metachromatic jelly layer of uterine eggs arises from the egg itself and not from the oviduct. It is of interest in this regard that homogenates of *Rana pipiens* ovarian eggs prepared late in the spring were often rendered useless as sources of platelets by a massive gummy precipitate which appeared during the first centrifugation.

b. Nature of the platelet surface

Staining reactions of fresh preparations indicated the existence of some sort of surface material different from the platelet interior. Not only did the surfaces of washed platelets stain more intensely with toluidine blue than did platelet interiors, but also toluidine blue-stained residues of lysed platelets (platelet ghosts) were found enmeshed in "ghost" material. These staining reactions support the evidence for the existence of a surface coat on platelets as found in micromanipulation and lysis experiments.

Staining reactions of platelets fixed *in ovo* also showed a surface coat, which was markedly stained by PAS, iron hematoxylin, basic fuchsin and acid fuchsin. However, the stained sections also revealed a tendency for cytoplasmic material to aggregate around yolk platelets, leaving large, optically clear spaces between platelets. Fixed extruded platelets (platelets released from oocytes cut during dissection and carried along with ovarian material through fixing and subsequent procedures) did not usually have as intensely a stained surface as did platelets fixed within oocytes. When a distinctly stained surface did occur on extruded platelets, these platelets were surrounded by cytoplasmic material which had been extruded along with the platelets from cut oocytes. Thus, the evidence suggests that the intensely staining coats of platelets fixed *in ovo* were produced by precipitated cytoplasmic components as a result of fixing techniques. Since both extruded platelets and washed platelets might also acquire similar surface precipitation coats during crushing, homogenization and washing procedures, even the occurrence of surface staining for such platelets would not prove that a coat exists for platelets in viable eggs. Of course, staining reactions leave unanswered any questions concerning a submicroscopic surface membrane.

c. Feulgen-positive yolk platelets

In addition to the Feulgen-positive nuclei of "ghost" material and ovarian membranes, a faintly positive response was noted for platelets in sectioned oocytes. Hibbard (1928) and Brachet (1950) previously reported a positive Feulgen reaction for amphibian yolk, but even in the absence of acid hydrolysis. However, results reported here for the yolk of *Rana pipiens* showed a greatly reduced response when acid hydrolysis was omitted. It is, nevertheless, open to question whether this Feulgen reaction of yolk is caused by DNA or is an artifact. The presence of non-nuclear DNA or DNA-precursors has been demonstrated for the amphibian egg by a number of investigators (Kutsky, 1950; Hoff-Jørgensen and Zeuthen, 1952; Sze, 1953; Gregg and Løvtrup, 1955; Finamore and Volkin, 1958; Grant, 1958; Bieber, Spence and Hitchings, 1959). But, according to Gregg and Løvtrup, the quantity of DNA that might exist in the cytoplasm of amphibian eggs could not be demonstrated by the Feulgen method. Even if caused by the presence of DNA, however, the weakly positive Feulgen response of yolk platelets in fixed oocytes does not unequivocally indicate the presence of DNA in platelets *in vivo*. It is possible that DNA or DNA-precursors diffuse into the platelets during fixation, as is suggested by the absence or diminution of the Feulgen stain in washed platelets and in extruded platelets.

3. *Electron microscopy of platelets and YC-fragments*

a. Nature of the platelet surface

Although some authors have presented electron micrographic evidence for the existence of an outer membrane on amphibian yolk platelets (Eakin and Lehmann, 1957; Wischnitzer, 1957; Karasaki and Komoda, 1958), the existence of such a membrane for mature platelets *in ovo* is questionable. Except for the possibility (certainly not to be disregarded) of membrane removal during isolation and preparation procedures, the failure to find a membrane for isolated platelets as reported here indicates that a differentiated surface coat may not be a normal feature for platelets *in ovo*. It is of interest that the membranes reported by other investigators were not a constant feature in their preparations. Since all the previous investigations were conducted on platelets fixed intracellularly, the membranes observed in these preparations might be surface-precipitated cytoplasmic material. In regard to yolk in embryos, platelets in embryonic ectodermal cells may be subject to surface alteration and enzyme deposition which would introduce another source of confusion. An electron-dense surface was sometimes visible on platelets in the study reported here, but this surface appeared to be similar or identical to the rest of the yolk platelet in periodic structure. It is possible that this electron-dense layer represents the coat found in micromanipulation and lysis studies.

b. Periodic structure of yolk

The existence of a periodic structure for yolk platelets has been reported for a number of amphibian species including *Triturus viridescens* (Wischnitzer, 1957), *Triturus pyrrhogaster* (Karasaki and Komoda, 1958) and *Rana pipiens* (Ward, 1959). The periodicity reported for the urodele forms, 70 Å, is similar to the periodicity for platelets of *Rana pipiens* as noted by Ward (personal comunication) and as reported here, 72 Å. A periodic organization of yolk components has also been reported for some invertebrates, including *Limnea stagnalis* (Wischnitzer, 1957) and *Planorbis corneus* (Favard and Carasso, 1958). It is likely that an ordered, periodic structure will be found for the yolk of many other organisms, especially in those forms with dense, crystal-like platelets.

The 72 Å-spaced lamellae found in the isolated yolk platelets of *Rana pipiens* were oriented approximately parallel to the long axis of the platelet. As also noted by other investigators for their preparations, these lamellae seem to consist of units closely packed along the length of the bend. Although electron micrograph resolution did not usually permit a clear definition of these units, near the edge of the platelet the bands were shown to be composed of individual units (Fig. 19).

The appearance of a somewhat irregular periodic structure and close packing for yolk in YC-fragments showed the similarity of YC to yolk platelets. This orientation of yolk components in YC-fragments may be important in the consideration of yolk platelet synthesis from yolk-like substances carried in the plasma of female frogs. Since yolk components can be easily precipitated from solution *in vitro* to form a densely packed YC material similar in organization to the yolk platelet, it is possible that yolk platelet synthesis is primarily a localized precipitation of yolk components following their transfer from the plasma to the growing oocyte.

SUMMARY

1. Yolk platelets of *Rana pipiens* ovarian eggs were studied by micromanipulation, lysis, histological and electron microscopic techniques. Investigations were carried out on washed, isolated platelets; unwashed, extruded platelets; and platelets *in ovo*.

2. The presence of a surface coat was demonstrated for isolated, washed platelets by micromanipulation and lysis. However, the evidence suggested that this coat might be a preparation artifact. Histological studies also showed a surface coat for platelets *in ovo*, but evidence indicated that this coat too was an artifact.

3. "Ghost" material (the insoluble residue of platelet suspensions after lysis) was found to be largely made up of follicular and other ovarian membrane fragments.

4. Yolk platelets fixed *in ovo* were faintly Feulgen-positive.

5. Electron microscopy of osmium-fixed, washed yolk platelets showed a periodic arrangement of yolk components (72 Å periodicity of 36 Å-wide lamellae or bands of closely spaced units). An electron-dense surface was noted on some platelets, but this "coat" showed the same periodic structure as the rest of the platelet.

6. Electron microscopy of YC-fragments (platelet-like material prepared from soluble yolk by a simple precipitation process) showed a close packing of yolk components similar to the packing found for yolk platelets.

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ORGANIZATION AND COMPOSITION OF THE AMPHIBIAN YOLK PLATELET. II. INVESTIGATIONS ON YOLK PROTEINS[1]

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As indicated elsewhere (Ringle and Gross, 1962), an understanding of the organization and composition of yolk platelets is a prerequisite for an adequate appreciation of the factors involved in yolk synthesis and utilization. Since amphibian yolk platelets are primarily lipo- and phosphoprotein structures, information pertaining to the composition and behavior of the protein components is important for understanding the make-up of the intact platelet.

The results reported here include investigations on both the soluble and insoluble fractions derived from lysed platelet suspensions of *Rana pipiens*. These fractions were studied by a number of chemical and immunological methods.

MATERIALS AND METHODS

Washed yolk platelets were prepared from ovarian eggs of *Rana pipiens* by the homogenization-centrifugation method of Essner (1954) as previously described (Ringle and Gross, 1962).

1. *Solubilization of yolk platelet proteins*

Yolk platelets were lysed by lytic concentrations of monovalent or divalent cation salts, usually 0.4–0.5 *M* NaCl or 0.5 *M* $CaCl_2$. The insoluble residue ("ghost" material) of washed platelet suspensions after lysis was removed by centrifugation at 700 × *g*. Precipitation of solubilized yolk proteins was accomplished either by direct dilution with or by dialysis against hypotonic solutions (dilute saline or distilled water). Precipitates and supernatants were separated by centrifugation. A summary of the soluble, insoluble, and precipitate fractions derived from washed platelet suspensions is given in Figure 1.

2. *Extraction of lipids*

NaCl-solubilized yolk (YP_{na}) in 0.5 *M* NaCl was lipid-extracted by a method similar to one described by Macheboeuf (1953) for the extraction of lipids from plasma lipoproteins. YP_{na} was lipid-extracted with an equal volume of ethanol: ether (9.6% ethanol by volume) by vigorously shaking 50–80-ml. volumes of YP_{na} with ethanol: ether in stoppered separatory funnels. These solutions were thus

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shaken for 20–30 seconds three times at approximately 30-minute intervals. Following extraction the saline (containing lipid-extracted yolk, YP_{df}) and ether fractions were separated and refrigerated for future analysis.

Carotenoids from the ether fraction were characterized by the analytical method described by Fox (1953) for the separation of carotenes and xanthophylls. The separation of these carotenoids is based on their differential solubilities in petroleum ether and in 90% methanol. Photometric analyses (optical density readings) of the carotenoids dissolved in petroleum ether were performed with a Beckman DU model 2400 spectrophotometer.

3. *Electrophoresis*

Precipitated soluble yolk fractions YP, YC, WC and YP_{df} (for the method of preparation see Results, 1.) were dissolved in pH 10.1 glycine-NaOH buffer and separately dialyzed under refrigeration for 24–48 hours against one liter of the buffer used for solubilization. Portions of these buffer-yolk solutions were refrigerated in stoppered containers and conductivity measurements performed 5–9 weeks later with a Leeds and Northrup conductivity bridge. Conductivity measurements showed almost identical conductivities for the stored solutions but, since these solutions had been stored, the conductivity values were not used to calculate protein mobilities. However, the similarity of the conductivity values made it reasonable to compare the number, size and distribution of peaks among the various runs. Electrophoretic analyses were performed with a Perkin-Elmer Model 38A electrophoresis apparatus equipped with a Rayleigh fringe accessory. Runs were conducted at 1.5° C. and 7.5 ±0.5 milliamperes.

4. *Nitrogen and phosphorus determinations*

a. Total nitrogen

Total nitrogen values were determined by the Kjeldahl method, using an Aminco-Koegel rotary digestion apparatus and an Aminco-Koegel glass distillation apparatus. Digestions were performed in dual-purpose flasks for 4 or more hours with concentrated H_2SO_4 plus selenium catalyst. Digested samples plus 5 ml. of 50% NaOH and 7 ml. of distilled water were distilled for 20 minutes. The distillate was collected in 10 ml. of 2% boric acid containing 8–9 drops of indicator (a 2:1 mixture of 0.1% bromocresol green and methyl red solutions). Titrations were done with 0.0098 N HCl. All samples were run in duplicate.

b. Total phosphorus

The procedure used was modified from methods described by Hawk, Oser and Summerson (1954) and Leloir and Cardini (1957). Digestions were performed in 30-ml. Kjeldahl flasks heated on an Aminco-Koegel rotary digestion apparatus. Flasks containing 0.5–1.0 ml. of test material and 1.0 ml. of 5 N H_2SO_4 were heated for 70 minutes. After cooling, two drops of 30% hydrogen peroxide (Superoxol) were added and the flasks heated for 20 minutes. Several milliliters of distilled water were then added and the flasks again heated for 20 minutes. After cooling 1.0 ml. of 2.5% ammonium molybdate was added to each flask and the contents then quantitatively transferred to graduated 12-ml. conical centrifuge tubes with three distilled water washes. Additional distilled water was added to bring the tube contents up to 9.8 ml. Reducing solution was prepared shortly before use by dissolving, in 10 ml. of distilled water, 250 mg. of a stock dry mix composed of 0.2 gm. 1-amino-2-naphthol-4-sulfonic acid, 1.2 gm. sodium bisulfite and 1.2 gm. sodium sulfite. To each tube was added 0.2 ml. of reducing solution and, 10 minutes after mixing, optical density values read at 660 mμ with a Bausch and Lomb Spectronic "20" spectrophotometer. The sample phosphorus content (μM P/sample) was determined from the o.d. readings by reference to a standard curve. All samples were run in duplicate.

5. *Nucleic acid determinations*

DNA (deoxyribonucleic acid) and PNA (pentose nucleic acid) were extracted from "ghost" and yolk samples with hot trichloroacetic acid by the method described by Schneider (1957). Most extractions were performed in 12-ml. conical glass centrifuge tubes, although in some cases 40-ml. tubes were used to accommodate larger samples (for which proportionately larger volumes of reagents were used for extraction). DNA-P and PNA-P in the extracts were determined using diphenylamine and orcinol reagents as described by Schneider. Although DNA and PNA standards were run and standard curves plotted, the values reported here were calculated for nucleic acid phosphorus by the formula given by Schneider. DNA-P and PNA-P values were calculated from the averaged o.d. readings of duplicate runs.

6. *Antigenic analyses*

a. Production of antisera

Soluble yolk antigen and "ghost" antigen were prepared by lysing yolk platelet suspensions with 0.4 M NaCl or with 0.5 M $CaCl_2$ + 0.1 M NaCl. "Ghost" material was separated from the soluble yolk fraction by centrifugation and washed 3–4 times in lytic salt solutions to remove soluble yolk. Soluble yolk and insoluble "ghost" fractions were stored separately under refrigeration prior to their injection as antigens. Protein concentrations of the antigen solutions were estimated from the volume of packed platelet material used for lysis (by the biuret method it was found that one ml. of packed platelets yields about 375 mg. of soluble protein). To produce antisera for the tube double diffusion tests, the protein concentration was approximately 28 mg. per ml. whereas a protein concentration of about 10 mg. per ml. was used to produce antisera for the Petri dish double diffusion test. "Ghost" protein concentration was one mg. per ml. Antigens were injected intravenously, one ml. per injection, with a total of 5–8 injections per animal, spaced 3–4 days apart. Albino rabbits (mixed sexes) and New Hampshire Red roosters were used. Injection, bleeding (intracardiac puncture), and serum preparation techniques were as described by Kabat and Mayer (1948). Sera were either used immediately after preparation or were shell-frozen in vials in an acetone-dry ice mixture and stored in a freezer for future use.

b. Tube double diffusion method

Antigen-antibody analyses in tubes were done in rimless culture tubes (10 × 75 mm.) which had been internally coated with a thin layer of agar as described by Munoz and Becker (1950). Solutions were introduced into these tubes with hand-drawn glass pipettes fitted to one- or two-ml. syringes by means of surgical rubber couplings. Serum (0.75 ml.) was overlayed with 2.0 ml. of warm (50–55° C.) 1% agar (Difco). After a brief refrigeration to hasten solidification of the agar, 0.75 ml. of antigen was added over the agar. The tubes were tightly stoppered with corks and stored at room temperature. Tubes were examined every other day for band formation (*i.e.*, antigen-antibody precipitates in the agar column).

All solutions used (agar, serum and antigen) contained 10 μg. of chloromycetin per ml. Other substances added are indicated in Table IV. Agar pH was adjusted to 7.0 ± 0.1 with 0.2 N NaOH.

c. Petri dish method

Petri dishes for the Ouchterlony method were filled with 75 ml. of 1% agar and refrigerated briefly. Wells to contain the reactants were made by forcing three 19-mm. diameter glass cylinders into the agar bed and were spaced 2 cm. apart. The agar within each cylinder was removed with a medicine dropper, leaving a thin layer of agar at the bottom of each well. Sera or antigen solutions were added to each well, the glass cylinders withdrawn, and the completed dishes covered and stored under a bell jar to reduce evaporation. All reagents used contained 1 : 10,000 Merthiolate. Agar pH was initially adjusted to 7.0 with 0.2 N NaOH and was finally made up in 0.05 *M* Tris buffer (pH 7.2) containing 2.0 *M* NaCl and 0.002% methyl orange. Sera contained 2.0 *M* NaCl and antigen solutions were either in 0.4 *M* NaCl or 0.05 *M* $CaCl_2$ (see Figure 4).

RESULTS

1. *Solubility of yolk platelets and yolk "fractions"*

The studies on the solubility of yolk platelets and yolk "fractions" precipitated from solubilized yolk are summarized in Figure 1.

a. Solubility in $CaCl_2$

Yolk platelet suspensions were lysed in 0.05 *M* $CaCl_2$, although both higher and lower concentrations of this salt will also result in platelet lysis (Holtfreter, 1946; Terry, 1950; Essner, 1954; Gross, 1954). Lysis in 0.05 *M* $CaCl_2$ resulted in a rapid dissolution of soluble yolk, leaving behind an insoluble "ghost" material which could be readily removed by low speed centrifugation. The "ghost"-free calcium-lysed yolk (YP_{ca}) was not as optically clear as NaCl-lysed yolk (YP_{na}), as has been previously reported (Gross and Gilbert, 1956). This mild turbidity was also noted in NaCl-lysed yolk solutions containing 0.05 *M* $CaCl_2$. No visible reduction in turbidity followed centrifugation at 41,150–56,500 × *g* for 15 minutes, thereby indicating that the turbidity was caused by particles less than 1000 Å in diameter. YP_{ca} was more easily surface denatured than YP_{na}, the former readily yielding stringy precipitates upon shaking or stirring. Also, refrigeration of YP_{ca} for 1–2 days was accompanied by a spontaneous precipitation of some flocculent material which was not noted in similarly treated YP_{na}.

Although prolonged exposure to calcium ions causes amphibian yolk to become permanently soluble, dilution with or dialysis against distilled water soon after

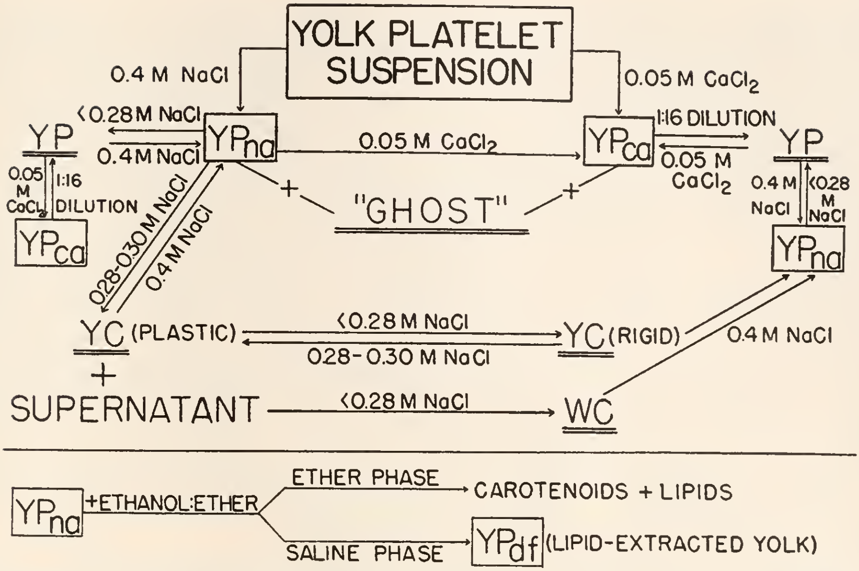
YOLK PLATELET SUSPENSION
0.4 M NaCl
0.05 M CaCl2
<0.28 M NaCl
0.4 M NaCl
YP
YPna
0.05 M CaCl2
YPca
1:16 DILUTION
0.05 M CaCl2
YP
0.05 M CaCl2
1:16 DILUTION
YPca
"GHOST"
+
+
0.4 M NaCl
<0.28 M NaCl
YPna
0.28-0.30 M NaCl
0.4 M NaCl
YC (PLASTIC)
<0.28 M NaCl
0.28-0.30 M NaCl
YC (RIGID)
0.4 M NaCl
+
SUPERNATANT
<0.28 M NaCl
WC
YPna
+ETHANOL:ETHER
ETHER PHASE
CAROTENOIDS + LIPIDS
SALINE PHASE
YPdf
(LIPID-EXTRACTED YOLK)

FIGURE 1. Soluble and insoluble fractions derived from washed yolk platelet suspensions. Detrital "ghost" material formed from both NaCl- and $CaCl_2$-lysed suspensions.

lysis promotes yolk precipitation (Flickinger, 1956). This finding was confirmed in the studies reported here. Both freshly lysed and refrigerated (up to three hours or more) YP_{ca} in 0.05 M $CaCl_2$ could be readily precipitated by 1:16 dilution with distilled water (one part YP_{ca} + 15 parts distilled water). Dilution 1:8 did not cause a precipitation of the solubilized yolk, thus indicating the critical concentration of $CaCl_2$ required for solubilization of *Rana pipiens* yolk to be near 0.003 M.

There was a marked difference in the kinetics of precipitation of soluble yolk (YP) from YP_{na} and YP_{ca} solutions: dilution of YP_{na} to a low salt concentration (0.2 M or less NaCl) resulted in a rapid precipitation of all the YP, whereas 1:16 dilution of YP_{ca} in 0.05 M $CaCl_2$ showed several seconds delay before the initial appearance of YP. Moreover, YP continued to accumulate for a minute or more after the 1:16 dilution. Precipitation seemed to be complete in 30 minutes or less, as judged by the failure of the resulting supernatant to yield further precipitate either by further dilution with distilled water or by the addition of an equal volume of 10% TCA.

b. Fractional precipitation of NaCl-lysed yolk (YP_{na})

Lysis of washed yolk platelet suspensions in 0.4 M or higher concentrations of NaCl yielded optically clear, yellow YP_{na} and an insoluble "ghost" material readily packed by centrifugation. This "ghost" fraction was similar in appearance to the "ghost" fraction resulting from lysis by $CaCl_2$. As previously reported (Ringle and Gross, 1962), the "ghost" fraction was found to consist largely of detrital remnants of ovarian and follicle membranes, together with some insoluble platelet material.

Precipitation of yolk from YP_{na} by dilution gave either of two physically different types of precipitate, depending upon the salt concentration of the medium in which the precipitation occurred. At salinities below 0.28 *M* NaCl a light, flocculent, creamy-white material (YP) formed; at 0.28–0.30 *M* NaCl there appeared a dense, viscous precipitate (YC). A proportionately small quantity of YP-like precipitate (WC) formed upon further dilution of the supernatant from YC preparations. At room temperature the dense YC material was yellow and transparent, much resembling honey in both appearance and fluidity. At low temperatures (3–5° C.) YC became opaque and rigid (Ringle, in press). YC also became opaque and rigid at room temperature if transferred to NaCl concentrations below 0.28 *M*, resulting in the formation of a dense material which could be broken up to yield irregular platelet-like YC-fragments. These YC-fragments behaved like platelets in lysis and micromanipulation experiments, and they also showed a relatively orderly and dense packing of yolk components as revealed by electron microscopy (Ringle and Gross, 1962). YC material occupied the same volume as the packed yolk platelets used to produce it (Table I).

After the precipitation of YP or WC material, no detectable proteins remained in solution, as indicated by the failure of further precipitate to form upon dilution of the supernatant with an equal volume of 10% TCA.

Although YP and YC were markedly different in appearance, they behaved similarly in lytic salt solutions. Furthermore, either YP or YC could be precipitated from redissolved YP or YC by reduction of the salt concentration to the proper level (0.28–0.30 *M* NaCl for YC, below 0.28 *M* for YP). YP precipitated from lysed yolk in 0.05 *M* $CaCl_2$ by 1 : 16 dilution with water could also be converted to either YC or YP by re-lysing in NaCl and subsequent dilution to the proper salt concentration. As indicated below, YP- and YC-type precipitates could also be produced from lipid-extracted YP_{na}.

2. *Extraction of lipids from* YP_{na}

Extraction of lipids from NaCl-lysed yolk by 9.6% ethanol : ether removed about 10% (9.0–11.1%) of the dry weight of YP. The extracted lipid contained all or most of the carotenoid pigment of yolk, since lipid-extracted yolk protein was white in color and did not form yellow solutions when dissolved in saline. Carotenoid pigment was largely xanthophyllic, since practically all of the yellow

TABLE I

*Volumetric analysis of yolk platelet suspension and its derivative YC, WC and "ghost"**

Item	Volume (ml.)
1. Washed yolk platelet suspension	4.90
2. YC (in 0.3 *M* NaCl)	4.00**
3. "Ghost" material	0.75
4. WC (in 0.1 *M* NaCl)	0.05
Total of items 2, 3 and 4	4.80

* Materials packed in graduated tube by centrifugation.

** This value slightly lower than its true value because a small portion of the sticky YC could not be removed from the dialysis tubing.

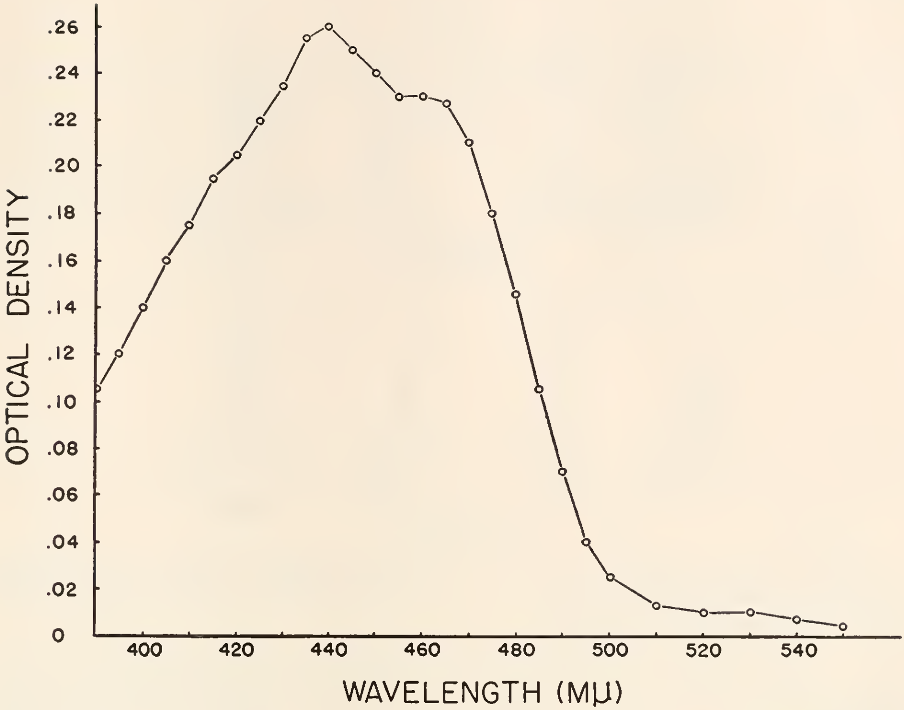

FIGURE 2. Absorption spectrum of yolk xanthophylls in petroleum ether. Absorption maximum at 440 mμ.

pigment was preferentially soluble in 90% methanol with only a small portion remaining in the petroleum ether epiphase. Photometric analysis of the methanol hypophase carotenoids redissolved in petroleum ether showed a light absorption maximum near 440 mμ (see Figure 2).

Except for a small gel-like fraction (presumably denatured yolk protein) which collected at the saline-ethanol: ether interface, most of the lipid-extracted YP_{na} material (YP_{df}) remained soluble in lytic concentrations of NaCl. YP_{df} retained the solubility properties of YP_{na} and, upon dilution, could be precipitated as YP- or YC-like precipitates at the proper salt concentrations. However, the YC-like precipitate in 0.3 *M* NaCl, although dense and viscous, was not yellow and transparent like normal YC. Also, it was easily scraped or poured from dialysis tubing, whereas YC was very sticky.

3. *Electrophoresis*

As indicated in Figure 3, all the yolk fractions tested exhibited two main electrophoretic peaks: a smaller, faster component (A) and a larger, slower component (B). The descending patterns showed in addition a variable number of minor peaks which may represent additional electrophoretic species. The trailing margin of the larger B component of YP_{df} showed some asymmetry, which suggests

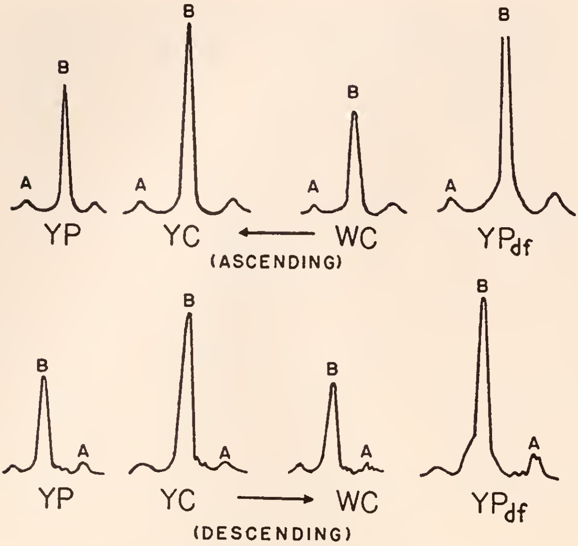

FIGURE 3. Electrophoretic patterns of soluble yolk fractions. Faster A and slower B components present in all fractions tested. Slow trailing peak (not labeled) an artifact and does not represent a yolk component.

that this peak was made up of at least two fractions. The stationary peak trailing the B peak in all runs is an artifact and does not represent an electrophoretic component.

4. *Phosphorus/nitrogen ratios*

Phosphorus/nitrogen (P/N) ratios as well as some dry weight/nitrogen ratios are given in Table II. Whole washed platelet suspension, YP (with one exception), and WC gave identical P/N ratios of 0.14. The P/N ratios for a single batch of YC and YP were slightly lower (0.13), and values as low as 0.093 were obtained for lipid-extracted YP (YP_{df}). "Ghost" material gave much higher P/N ratios, 0.20–0.21. Aging of yolk platelets and YP under refrigeration (up to 21 days), and dialysis of YP against refrigerated distilled water for 4 days, did not alter yolk P/N ratios.

Nitrogen determinations on the total "ghost" and soluble yolk portions of three different batches of washed yolk platelets showed that the "ghost" fraction represented a fairly constant proportion of the total washed preparation (Table III). This finding indicates that the homogenization-centrifugation technique for isolating yolk may be relied on to produce similar yolk preparations from different batches of *Rana pipiens* ovaries.

5. *Nucleic acids*

Nucleic acid determinations for various yolk and "ghost" samples are summarized in Table II as nucleic acid phosphorus/nitrogen ratios. "Ghost" material gave the highest DNA-P/N (0.011–0.026) and PNA-P/N (0.0038–0.012) ratios.

TABLE II

Chemical analyses on yolk platelets and yolk fractions

Item	Phosphorus/nitrogen (P/N) ratio	Dry wt./nitrogen ratio	DNA-P/N ratio	PNA-P/N ratio
Yolk platelet suspension	0.14 (2)*	—	0.00074	0.00062
YP	0.13 (1) 0.14 (4)	6.7	0–0.00095	0.00016–0.00072
YP_{df}	0.093–0.12 (3)	6.4–6.5	— —	— —
YC	0.13–0.14 (2)	—	—	—
WC	0.14 (1)	—	—	—
"Ghost"	0.20–0.21 (3)	6.3–6.6	0.011–0.026	0.0038–0.012
Supernatant**	—	—	0.00037	0.0017

* Numbers in parentheses ( ) indicate the number of batches of yolk analyzed.

** The supernatant from the first centrifugation after ovarian homogenization.

Lower ratios were found for whole washed platelet suspension, YP_{na}, YP precipitate, and the supernatant from the ovarian homogenate.

6. *Antigenic analyses*

Antigen-antibody analyses on yolk by the double diffusion method utilized a number of different salt, antigen and antibody concentrations. Some of the results of these investigations are summarized in Table IV (tube double diffusion method) and in Figure 4 (Petri dish method). The maximum number of bands of antigen-antibody precipitate found for solubilized yolk (both NaCl- and $CaCl_2$-lysed yolk) was five. At least three of the soluble antigens of yolk were also found in "ghost" material, since antisera to "ghost" material showed three zones of precipitate when reacted with soluble yolk.

TABLE III

Total nitrogen of insoluble "ghost" and soluble fractions of washed yolk platelet preparations

Preparation	"Ghost" N (mg.)	Yolk N (mg.)	Per cent "ghost" N
A	0.90	310	0.29
B	1.60	560	0.29
C	0.78	260	0.30

TABLE IV

Antigenic analysis of amphibian yolk by double diffusion in agar

Tube #	Antigens	Concentration of saline in agar	Antisera*	Maximum # of bands
1	Calcium-lysed YP in 0.4 *M* NaCl (protein conc. 0.7%)	0.4 *M* NaCl	A-1 (0.4 *M* NaCl)	5
2	Same as above	Same as above	A-2 (0.4 *M* NaCl)	3
3	Calcium-lysed YP in 0.15 *M* NaCl + 0.05 *M* $CaCl_2$ (1% protein)	0.15 *M* NaCl + 0.05 *M* $CaCl_2$	A-3	3
4	Same as above except concentration of protein 0.25%	Same as above	A-3	3**
5	NaCl-lysed YP in 2.0 *M* NaCl + 0.05 *M* $CaCl_2$ (1% protein)	2.0 *M* NaCl + 0.05 *M* $CaCl_2$	A-3 (2.0 *M* NaCl)	5
6	Same as #3 except lysed YP was frozen before use	Same as #3	A-3	4
7	NaCl-lysed YP in 0.4 *M* NaCl (1% protein)	0.4 *M* NaCl	A-3 (0.4 *M* NaCl)	4
8	NaCl-lysed YP in 2.0 *M* NaCl (1% protein)	2.0 *M* NaCl	A-3 (2.0 *M* NaCl)	3
Control tubes: bovine serum albumin vs. antiserum; antigens vs. normal serum				0

* Rabbit antisera: A-1, anti-YP (lysed in 0.4 *M* NaCl). A-2, anti-"ghost" (from NaCl-lysed yolk suspension). A-3, anti-YP (lysed in 0.05 *M* $CaCl_2$).

** Better separation of bands than in tube #3.

DISCUSSION

1. *Solubility of yolk platelets and yolk "fractions"*

The studies reported here show that the major fraction of washed yolk platelet suspensions is readily soluble in lytic concentrations of NaCl or $CaCl_2$. If this solubility is taken as a criterion that the yolk proteins are in their native state, then it would appear that the homogenization-centrifugation procedure for isolating yolk platelets leaves them in a state similar to that *in ovo*. The remarkable stability of yolk platelet proteins is further demonstrated by platelet solubility in lytic concentrations of NaCl and $CaCl_2$, even after storage under refrigeration for many weeks. Detectable denaturation of yolk particles, either platelets or YC-fragments, seems to be limited to alterations of yolk surfaces in contact with homogenization or suspension media (Ringle and Gross, 1962). The stability of yolk proteins of isolated platelets increases the likelihood that studies on washed platelets will yield information pertinent to an understanding of yolk platelets *in ovo*.

The effects of solubilization of yolk by sodium and calcium salts are quite different, even apart from the permanent solubilization of yolk proteins by pro-

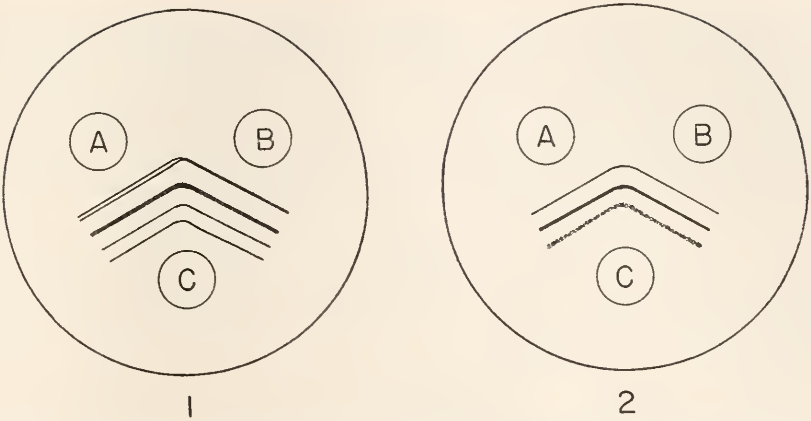

FIGURE 4. Ouchterlony Petri dish double diffusion method for the analysis of antigens in yolk and "ghost" material. Well contents: A, NaCl-solubilized YP; B, $CaCl_2$-solubilized YP; C, antiserum. Analysis 1: chicken antiserum (anti-YP_{ca}) in well C. Note five bands of antigen-antibody precipitate with YP_{na}, four bands with YP_{ca}. Analysis 2: rabbit antiserum (anti-"ghost") in well C. Controls (control serum in well C) negative for both analyses 1 and 2.

longed incubation with calcium ions. Differences in ionic strengths required for lysis, turbidity of the lysed yolk solutions, ease of denaturation, and kinetics of precipitation upon dilution all suggest a difference between the mechanisms of solubilization by NaCl and $CaCl_2$. Nevertheless, short exposure of solubilized yolk to calcium does not permanently alter its solubility characteristics, since the dilution precipitate of freshly calcium-lysed yolk can be dissolved in 0.4 *M* NaCl and will yield either YP or YC at the proper salt concentrations.

The different types of precipitate that form upon dilution of NaCl-lysed yolk (YP, YC, and WC) were at first thought to represent different protein fractions. However, since each of these "fractions" can be redissolved in 0.4 *M* NaCl and YP, YC and WC material produced from any one of these fractions, it is likely that they merely represent different physical states of the same yolk components. This apparent similarity is further verified by the electrophoretic analyses reported here.

Both YC and yolk platelets are dense materials, and YC was found to be identical in volume to the platelets from which it was derived. This similarity in volume indicates that the components of YC may be organized as in yolk platelets. The ability to transform viscous YC to a rigid, platelet-like material by a reduction in salt concentration also indicates a similar macromolecular packing in YC and platelets. Electron microscopy of YC-fragments and yolk platelets has shown a resemblance in the organization of these materials. A possible significance of this similarity between YC and platelets in relation to yolk synthesis has been suggested (Ringle and Gross, 1962).

2. *Extraction of lipids from YP_{na}*

Preliminary attempts to remove lipid from dried YP by extraction with hot ethanol: ether using a Soxhlet apparatus resulted in very poor yields of lipid.

Low lipid values with hot ethanol: ether have also been reported by Gross and Gilbert (1956). In view of the difficulty in extracting lipid from dried material, a method was used which enabled lipid to be extracted from fresh yolk in aqueous solution. The average value (10%) for lipid extracted with ethanol: ether solutions represents approximately 50% of the total yolk platelet lipid, based on yolk lipid values reported by Schjeide, Levi and Flickinger (1955). It is interesting that Macheboeuf (1953) was likewise able to remove only about half of the total serum lipids by a similar method of extraction with ethanol: ether.

Most or all of the carotenoids which impart the yellow color to amphibian yolk are extracted from YP_{na} by this method. It is likely, therefore, that the carotenoids are restricted to the more easily extracted lipid component of yolk lipoproteins. The preponderance of xanthophylls in YP agrees with the data reported by Fox (1953) for the carotenoids of frog ovaries.

Although removal of carotenoid-bearing lipid from yolk lipoproteins does alter the color and stickiness of YC made from defatted YP_{na} (YP_{df}), this removal of lipid does not noticeably alter the responses of yolk proteins to lytic concentrations of salts. The lytic properties of amphibian yolk seem, therefore, to reside with the protein or protein plus carotenoid-free lipid components.

3. *Electrophoresis*

The yolk fractions of *Rana pipiens* investigated here all showed two main electrophoretic peaks: a smaller, faster one and a larger, slower one (Fig. 3). This finding agrees with the results of other investigators on amphibian yolk. Flickinger and Nace (1952) found two electrophoretic fractions in the saline-soluble extract of mature *Rana temporaria* eggs. Barth and Barth (1954) also reported two fractions (a smaller, faster and a larger, slower one) in the KCl extract of *Rana pipiens* ovaries. They indicated the smaller fraction to be richer in phosphorus. Schjeide, Levi and Flickinger (1955) in studies on vitellin from washed platelets found one major component and a variable number of minor peaks preceding and trailing the main one. Two main yolk components have also been found by ultracentrifugation (Schjeide, Levi and Flickinger, 1955; Flickinger and Schjeide, 1957): a quantitatively smaller 6 S component rich in phosphorus and a larger 11 S component. Gross and Gilbert (1956) reported at least three fractions in *Rana pipiens* yolk by ultracentrifugation, with the main fraction showing a sedimentation value of 11 S. Thus both electrophoretic and ultracentrifugation studies have revealed two or more principal components in amphibian yolk.

Electrophoretic findings reported here concerning the occurrence of two peaks with similar mobilities and quantitative distribution support the idea that YP, YC, WC and YP_{df} are similar to one another. The slight asymmetry shown by the larger, slower electrophoretic peak of lipid-extracted YP_{df} indicates that this larger peak represents the principal lipid-bearing component of yolk.

4. *Phosphorus/nitrogen ratios*

As indicated in Table II, P/N ratios for "ghost" material are much higher than for whole yolk or yolk fractions (0.20–0.21 for "ghost" as compared with 0.13–0.14 for yolk platelets or unextracted soluble yolk). The higher P/N ratios of "ghost" material support the conclusion that this saline-insoluble fraction is largely non-yolk in origin (Ringle and Gross, 1962).

Extraction of lipids from soluble yolk results in lower P/N ratios for the extracted yolk (0.093–0.12). It was not determined whether the removed phosphorus was contained in the ethanol : ether or the saline fraction after lipid extraction. In reference to the lower P/N ratios of lipid-extracted yolk, it is interesting that Panijel (1950) found that small yolk platelets contain less lipid and have lower P/N ratios than do large platelets.

There are indications that species differences exist for the P/N ratios of amphibian yolk. The 0.14 P/N ratio reported here for washed platelets of *Rana pipiens* is intermediate in value between the ratios reported by Panijel (1950) for platelet suspensions of *Rana fusca* (0.145) and *Rana esculenta* (0.125). Lower P/N ratios of 0.080 and 0.113, respectively, were calculated for the yolk of *Rana pipiens* from data of McClendon (1909) and Gross and Gilbert (1956).

Similar P/N ratios were found here for whole yolk platelet suspension and unextracted yolk fractions. Also, P/N ratios of platelets and YP remained constant, even after prolonged storage and dialysis. Panijel (1950), however, found that his platelet preparations lost up to 30% of their phosphoprotein phosphorus in 24 hours unless refrigerated at 0° C. or less in 15% sucrose. According to Panijel, 70.6% of the yolk phosphorus is from phosphoprotein, which would mean a loss of over 21% of the total yolk phosphorus. Such losses would markedly alter P/N ratios. Perhaps differences in the methods of preparation of washed platelets account for the differences in phosphorus lability.

5. *Nucleic acids*

Nucleic acid-P/N ratios reported here show a marked variability in the nucleic acid content of the various soluble and insoluble yolk platelet suspension fractions. This variability is related, at least in part, to the nature of the fraction involved. Thus, "ghost" preparations show a relatively high DNA-P content, which correlates with the cellular detrital nature of the bulk of this material. Nucleic acid-P/N ratios of "ghost" material are within the range of values obtained for a number of cells and tissues by other investigators (Leslie, 1955). There is also variability in nucleic acid content of soluble yolk prepared from different batches of washed yolk platelets. DNA-P/N and PNA-P/N ratios ranged, respectively, from 0–0.00095 and 0.00016–0.00072. Bieber, Spence and Hitchings (1959) have reported that both seasonal and dietary factors affect the DNA-content of the eggs of *Rana pipiens*.

Large quantities of DNA or DNA-like material, ranging from 0.012 to 1.23 μg. per egg or early embryo of amphibians, have been detected by a number of investigators (Kutsky, 1950; Hoff-Jørgensen and Zeuthen, 1952; Sze, 1953; Gregg and Løvtrup, 1955; Finamore and Volkin, 1958; Grant, 1958; Bieber, Spence and Hitchings, 1959). From the data of the studies reported here the theoretically possible contributions of DNA by yolk and "non-yolk" fractions were calculated from their DNA-P/N ratios (Table II). These calculations were based on DNA- and PNA-phosphorus values suggested by Schmidt and Thannhauser (1945) and the 112 μg. of yolk nitrogen per egg of *Rana pipiens* as reported by Gregg and Ballentine (1946). Although variable in amount, the calculated DNA from soluble yolk ranged up to 1.08 μg. per egg, which is enough to account for the high DNA values of Kutsky (1950) and Sze (1953). In addition the "non-yolk" homogenate supernatant could itself supply a significant amount of DNA to the egg (0.18 μg.).

PNA-P values for the various yolk samples also showed a fairly marked variation, and some were much lower than 0.01, a ratio calculated for platelets of *Rana esculenta* from data of Panijel (1950). The higher PNA-P/N ratio for "non-yolk" homogenate supernatant is probably related to the presence of microsomes.

Unfortunately there are many factors that make it difficult to assign specific nucleic acid values to yolk and yolk fractions from homogenized eggs. The presence of "ghost" material in platelet suspensions introduces the possibility that some nucleoprotein is extracted from "ghost" nuclei during platelet lysis. However, the small quantity (0.3% or less) of "ghost" nitrogen in platelet suspensions and the short time involved in lysis would not indicate such an origin for the DNA of YP. The determination of normal nucleic acid values for yolk is also complicated by the prolonged washing procedures necessary for the preparation of relatively clean suspensions. Although the solubility of DNA-proteins is not marked at the low (0.1 *M*) NaCl concentration used for washing and storage of platelets (Frick, 1949), some nucleoprotein material could be lost during washing and storage. Possible surface adsorption of nucleoproteins during homogenization also complicates interpretation of results. Nevertheless, the occurrence of DNA in washed platelet suspensions and solubilized yolk corroborates the weakly positive Feulgen reaction shown by yolk platelets fixed *in ovo* (Hibbard, 1928; Brachet, 1950; Ringle and Gross, 1962).

6. *Antigenic analyses*

The results of double diffusion experiments reported here show that soluble yolk possesses a minimum of 5 antigens. This finding is compatible with the results of Cooper (1950) who reported 5–7 antigens for the eggs of *Rana pipiens*. Since she was dealing with eggs or egg extracts, a greater number of antigens might be expected than in washed yolk suspensions, which would account for the occurrence of as many as 7 antigens. Using calcium-lysed yolk prepared from washed yolk platelets to evoke antibody production in rabbits, Flickinger and Rounds (1956) found a maximum of 5 antigens in both calcium- and sodium-lysed yolk. They found that both types of lysed yolk yield the same band precipitate pattern in agar, thus indicating the antigenic similarity of these lysed preparations. The results of the Petri dish method reported here also indicate a similarity for the antigens of calcium- and sodium-lysed yolk, although two of the bands of sodium-lysed yolk formed only a single band with calcium-lysed yolk (Fig. 4).

Differences in the number of bands of precipitate formed during double diffusion does not necessarily indicate differences in antigen number. The effects of varying salt and protein concentrations on the number of bands formed by reacting antigen-antibody systems are evident from the results reported in Table IV. In addition to different numbers of bands, marked differences were sometimes noted in the appearance of bands from tubes containing the same antigens and antibodies (but different salts or salt concentrations). Thus, although tube #8 (Table IV) gave only three bands as opposed to five bands in tube #5, one of the three bands in tube #8 was much broader and presumably contained more precipitate than any single band in the other tube. It is suggested that the more prominent band contained precipitate which formed several bands in the presence of added 0.005 *M* $CaCl_2$. Although differences in tube contents caused differences in band formation, duplicate tubes always gave similar results. The differences in number and appearance of bands under different experimental conditions demonstrate that a variety of results can be obtained from the same antigens and antisera, depending upon the other reagents used. The effects of reagent concentration on band formation in agar have been discussed by Oudin (1952), Preer (1956), Preer and Telfer (1957) and Wilson (1958).

A number of different salt combinations and concentrations were tested because soluble yolk forms a precipitate upon diffusion into agar (at the agar-yolk interface), even when the agar contains 0.4 *M* NaCl. By increasing the NaCl content of agar to 2.0 *M* it is possible to eliminate this non-specific precipitate. Flickinger and Rounds (1956) also found this type of precipitate and that it could be prevented by adding 10% NaCl to the agar.

Freezing yolk prior to its use against antiserum promotes the formation of a larger number of bands than form from non-frozen yolk (Table IV, tubes #3 and #6). Also, bands from frozen yolk appear more rapidly than do bands from non-frozen yolk. In view of the damaging effects of freezing on lipoproteins (Lovelock, 1957), the change in band formation caused by freezing suggests that antigenic substances are thereby made more free to escape from or move through the non-specific yolk-agar precipitate.

Chicken antisera were prepared to determine if more precipitate bands would result than with rabbit antisera in agar containing 2.0 *M* NaCl. Goodman and Wolfe (1952) reported that maximum precipitation occurs with chicken antisera at 8–12% NaCl, whereas salt concentrations higher than 0.15 *M* NaCl reduce the amount of specific precipitate with rabbit antisera (Kabat and Mayer, 1948). The results with chicken anti-yolk antisera do not, however, reveal more than the maximum number of 5 bands found with rabbit antisera (Fig. 4).

Injection of suspended washed "ghost" material into rabbits causes the production of antibodies which react with at least three of the antigens present in soluble yolk (Table IV and Fig. 4). "Ghost" antigenicity could be caused by the normal presence of yolk antigens in the cellular material, by adsorption of soluble yolk during lysis, or by the insoluble ghosts (surface residues) of lysed platelets.

SUMMARY

1. Washed yolk platelet suspensions (prepared from ovarian eggs of *Rana pipiens*) lyse in the proper concentrations of NaCl or $CaCl_2$, resulting in solubilized yolk (YP_{na} or YP_{ca}) and an insoluble "ghost" residue. Different precipitation effects result with NaCl and $CaCl_2$ at lowered salt concentrations.

2. A compact precipitate (YC) resembling yolk platelets in density and volume results from a reduction of the salt concentration of YP_{na} to 0.28–0.30 *M* NaCl. A further reduction in salinity transforms YC from a plastic to a rigid material.

3. About 10% of the dry weight of soluble yolk is readily extracted with 9.6% ethanol: ether without affecting the solubility properties of the yolk proteins. The extracted lipids contain all or most of the yolk carotenoids, primarily xanthophylls.

4. Electrophoretic analyses show yolk fractions precipitated from YP_{na} by dilution (YP, YC, and WC) to be similar. Lipid-extracted yolk (YP_{df}) also gave a similar electrophoretic pattern.

5. Differences between the phosphorus/nitrogen ratios of whole yolk suspension and YP_{na} (0.14) and "ghost" material (0.20–0.21) support the conclusion that "ghost" material is largely non-yolk in origin.

6. Both DNA-P and PNA-P are detected in washed yolk platelet suspension and in soluble yolk, but the quantity found varies among different batches of yolk. The amount of DNA-P in some preparations is sufficient to account for the excess DNA/egg reported by previous investigators.

7. A minimum of 5 antigens are found for solubilized yolk, using both rabbit and chicken antisera. "Ghost" material shows three antigens in common with soluble yolk.

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RADIOACTIVE PHOSPHORUS ACCUMULATION AND DISTRIBUTION IN TETRAHYMENA[1]

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The protozoan, *Tetrahymena pyriformis,* has in recent years become increasingly important in biochemical and physiological protozoology (Corliss, 1954). Mating types I and II of variety four were used in this investigation. First defined by Elliott and Hayes (1953) and then more extensively by Ray (1956), the cytological picture of these types appears to be representative of conjugation in all 45 mating types of the nine known varieties of *Tetrahymena* (Gruchy, 1954). According to Ray (1956) there are three prezygotic and three postzygotic divisions of the micronuclear derivatives during conjugation in this animal. Meiosis occurs during the first two prezygotic divisions and mitosis during the third prezygotic and all of the postzygotic divisions. Morphological reduplication is thought to occur in the crescent stage when chromosomal material elongates during prophase of the first meiotic division.

Since nothing was known concerning ion accumulation and distribution during cellular differentiation in conjugating protozoans, the intent of this investigation was to determine the influence of conjugation and its nuclear reorganization on phosphorus accumulation. The isotope P^{32} was chosen primarily because of the role of phosphorus in DNA synthesis. We were especially interested in delineating ion movements and accumulation just prior to the formation of the crescent stage, since it is here that an increased P^{32} uptake for DNA synthesis with chromosomal reduplication might be expected (Swift, 1950). Taylor (1952), working with *Tradescantia* and *Lilium* buds, found that periods of P^{32} incorporation into nuclei corresponded with the interphase period of chromosomal reduplication and DNA synthesis in both mitosis and meiosis. Other work with metazoan animal cells (Hull and Kirk, 1950) has also shown a correlation of P^{32} uptake with the rate of mitosis. A differential rate of P^{32} uptake may also result from other reasons than chromosomal incorporation. Since phosphorus plays an essential role in energy transfer processes, it can be assumed that the total energy required at certain stages of the tremendous nuclear reorganization during conjugation might involve ion uptake in significant quantity.

It was further decided to investigate P^{32} uptake in mass populations of the individual mating types in an attempt to evaluate better the comparative amounts of this substance used by each type while population interactions were present.

Finally, in an attempt to evaluate further physiological differences between the

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two mating types, the quantitative intracellular distribution of phosphorus was studied. Thus, the distribution of phosphorus in the acid-soluble, phospholipid, nucleic acid and phosphoprotein fraction was investigated.

MATERIALS AND METHODS

The strains used were cultured in 250-ml. Erlenmeyer flasks containing peptone-tryptone medium (Slater, 1952). The initial pH was set at 7.6 and the length of incubation at 27° C. ±1° was 48 hours for log phase animals or 96 hours for those in the stationary phase. Each flask was initially inoculated with 10,000–20,000 cells.

Since conjugation in *Tetrahymena* can be induced by starvation in distilled water, the following procedure was used to produce masses of conjugating pairs. They were removed from the peptone medium by gentle centrifugation, washed twice with glass-distilled water and placed in glass-distilled water for 12 to 16 hours. They were then washed once again to free them from excretory products and energy sources, and reconcentrated. This was the preliminary procedure followed in all of the experiments.

The isotope used was P^{32} obtained as H_3PO_4 in weak HCl from the Oak Ridge National Laboratory. Specific activities of stock solutions averaged 60,000 mc./gm. The final concentration was 0.2 μc./ml. except where otherwise indicated. Radioactive organisms in the aliquots used for counting purposes were washed by centrifugation in construction chamber centrifuge tubes (Slater, 1957), the supernatant solutions being removed with micropipettes. Samples were then quantitatively transferred to 2.0-ml. volumetric test tubes and final volumes were adjusted to 2.0 ml. with distilled water. This volume was used to maintain constant geometry for counting. Finally, the samples were quantitatively transferred to 10-ml. plastic tubes.

Radiation counts were made with a deep-well scintillation detector and a TMC scaler (Model SG-2A) for all of the experiments except those having to do with the influence of population density and the phosphate distribution studies. For these later studies a Nuclear Chicago Ultra-Scaler was used. In all cases, 10-minute counts were taken, corrected for background, and calculated on a count/minute/animal basis. A self absorption study showed that corrections for this factor could be ignored.

For the conjugation studies, the two mating types after starvation were brought to equal optical densities by means of a Lumetron (Model 400) colorimeter at a wave-length of 650 mμ (Red). This made it possible to control the number of organisms used (Elliott, 1949). Equal concentrations of these solutions were then mixed, usually to a final volume of 100 ml. The P^{32} was then added, and this mixture was then distributed to 125-ml. Erlenmeyer flasks in 10-ml. aliquots. Samples were taken at this point for direct cell counts.

To test for P^{32} leakage, animals mixed for the induction of conjugation were allowed to take up the isotope for 9½ hours and then washed. Aliquots of the exposed animals in isotope-free distilled water were then counted for radioactivity at two-hour intervals.

With the individual mating types, the procedures were essentially the same as those outlined above for studying uptake during conjugation. Here, of course, the mating types were not mixed, and direct counts of animals were made of each of the types after first adjusting them to approximately the same population density.

Since population density has been shown to influence ion uptake in experiments with Co^{60} (Slater, 1957), the influence of concentration of organisms on P^{32} uptake in each mating type was investigated. For this study, cultures of each mating type were grown for two days in 250-ml. Erlenmeyer flasks containing 25 ml. of stock peptone medium. The cells were then harvested by mild centrifugation (100 × g for 45 seconds) and after washing twice with glass-distilled water were suspended in 50 ml. of water. This constituted the highest population density. Dilutions from this population at approximately 3.5×10^6 cells were then prepared. Radioactive phosphorus was introduced to the extent of 0.10 μc./ml. After nine hours, the cells were washed free of external radioactivity and by successive centrifugation were concentrated to 2.0-ml. quantities for counting in the scintillation detector.

To determine the intracellular distribution of phosphorus in each mating type, cultures were grown for 48 hours in 250-ml. Erlenmeyer flasks, each containing 100 ml. of stock peptone medium. The protozoans were then removed and washed by means of further centrifugation with glass-distilled water. The suspensions were pooled and then diluted to 10 ml. with distilled water. Aliquots were removed for counts of radioactivity. Samples were also removed for cell counts.

The procedure used for the isolation of the general classes of phosphorus compounds was modified from Moraczewski and Kelsey (1948), and approximately 2×10^6 animals were used for each determination. Cell suspensions were initially placed in 15-ml. conical centrifuge tubes and 10 ml. of 15% trichloroacetic acid were added per 5 ml. of cell suspension. These were shaken for 15 minutes and then centrifuged at 150 × g for 5 minutes. This thoroughly packed the sediment and resulted in the production of a clear supernatant liquid containing the acid-soluble fraction. The volume of liquid was recorded and 2.0 ml. were removed for radioactivity counts. The remaining liquid was then removed. One-half ml. of distilled water was added to the residue and after shaking for 15 minutes, 10 ml. of 3:1 alcohol-ether were added. This was then allowed to stand overnight, shaken 15 minutes and centrifuged 5 minutes. The soluble phospholipids present in the clear supernatant were then removed by pipette, the total liquid volume recorded, and 2.0-ml. aliquots removed for determinations of radioactivity.

Nucleic acid phosphorus was determined by adding 3 ml. of distilled water, shaking 15 minutes, boiling two minutes, and then adding 10 ml. of 10% trichloroacetic acid, followed by boiling again for 10 minutes. After cooling, the suspension was centrifuged 5 minutes. The dissolved nucleic acids were then removed quantitatively and 2.0-ml. aliquots taken for counts.

The determination of phosphoprotein phosphorus was made by adding 7 ml. of 0.2 N NaOH to the residue remaining after removal of the dissolved nucleic acids. This was then centrifuged, the total volume recorded, and aliquots were taken for radioactivity referable to phosphoprotein phosphorus in the solution.

RESULTS

P^{32} uptake during conjugation. At relatively low population densities (about 11,000 animals/ml.) there was a significantly increased uptake of P^{32} six hours

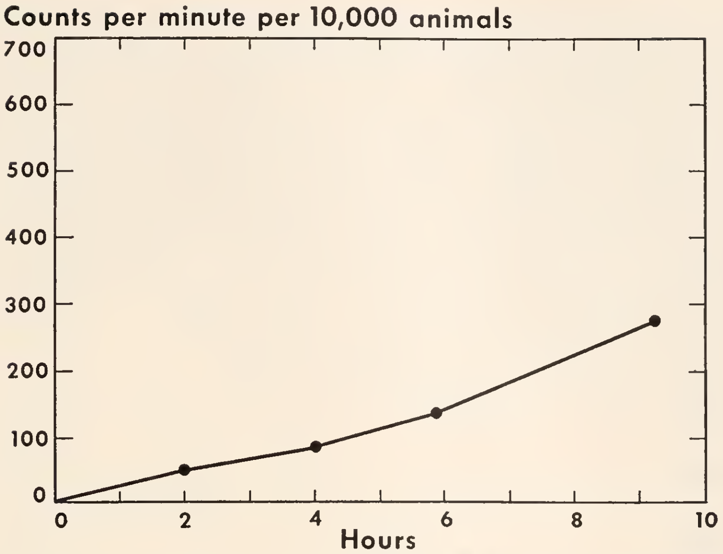

FIGURE 1. Uptake of radioactive phosphorus in *Tetrahymena* at low population densities. Maximum conjugation 30%. Average concentration of animals 11,000/ml.

after mixing the mating types for conjugation (Fig. 1). Feulgen staining of cultures after six hours indicated that the crescent stage of micronuclear division was prominent in all of the conjugating pairs. Prezygotic, meiotic divisions occurred between six and nine hours. At this relatively low population density, however,

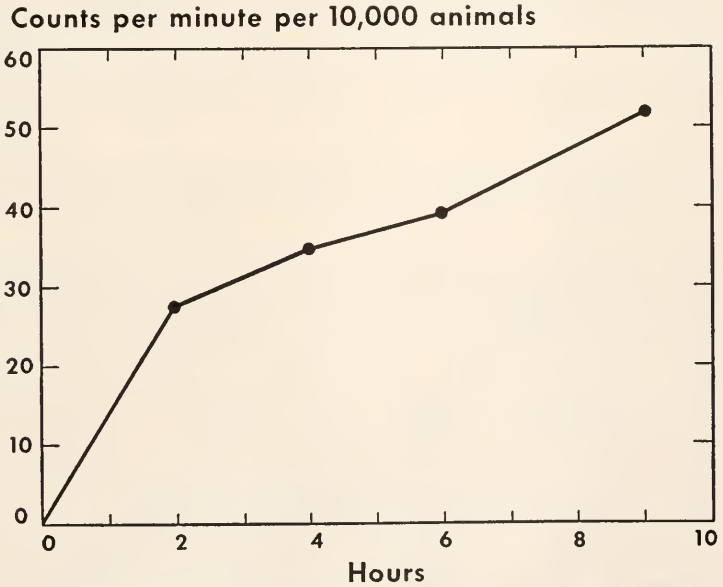

FIGURE 2. Uptake of radioactive phosphorus in *Tetrahymena* at high population densities. Maximum conjugation 60%. Average concentration of animals 236,000/ml.

conjugation at nine hours was less than 30% and synchrony of division was also low.

At higher population densities (Fig. 2) (236,000 animals/ml.) the rate of uptake of P^{32} remained constant at 3×10^{-4} cpm/animal/hr. after two hours. This was less than one-seventh that found when lower population densities were used. Up to 60% conjugation occurred when population densities were greater than 200,000 cells/ml. Further studies have also shown that population densities higher than about 260,000 cells/ml. result in reduced conjugation. A differential rate of uptake as seen with lower populations may have been masked by a superimposed population density effect, however, since it was not possible to correlate any uptake rate difference with a particular nuclear stage.

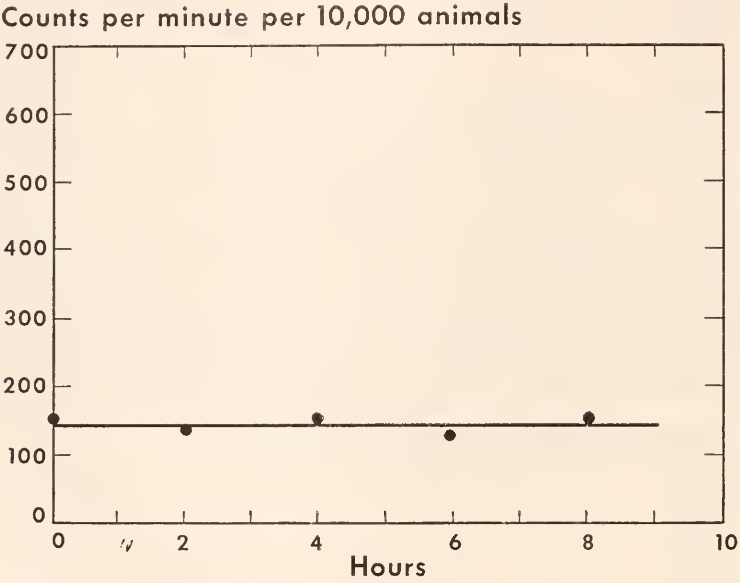

FIGURE 3. P^{32} retention in *Tetrahymena* when placed in distilled water after incubation with radioactive phosphorus. Concentration of animals 54,000/ml.

Leakage of P^{32} from conjugating Tetrahymena. The results of experiments in which all supernatant P^{32} was washed from conjugating animals, where radioactivity was measured at intervals, showed that leakage of phosphate from the ciliates could be eliminated as a possible source of error in the conjugation experiments. As Figure 3 shows, there was no loss of labeled phosphate from animals exposed to P^{32} when they were later placed in distilled water for periods up to eight hours.

P^{32} uptake in the individual mating types. When the individual mating types, after a starvation period, were tested for the uptake of P^{32} in distilled water, it was found that there was a significant difference between the two. The population densities for mating type I varied in the different experiments from 44,000 animals/ml., while those for mating type II varied from 34,000 animals/ml. to 244,000 animals/ml. Errors in these counts amounted to less than 5%.

It is evident that at these population densities mating type II takes up con-

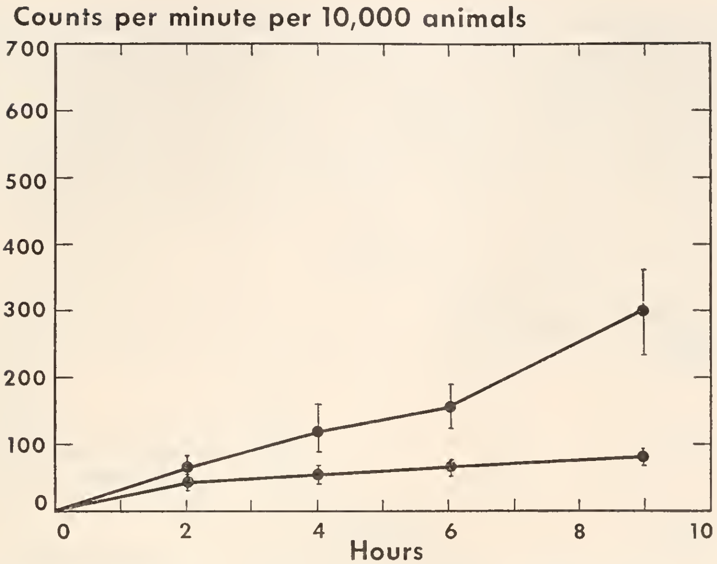

FIGURE 4. P^{32} uptake in the individual mating types. Mating type I. Lower curve. Average concentration: 100,000 animals/ml. Mating type II. Upper curve. Average concentration: 106,000 animals/ml. Standard deviation indicated.

siderably more P^{32} than mating type I and shows also an increased rate of uptake at six hours (Fig. 4). Mating type I, on the other hand, typically showed a slow, constant rate of uptake. It can readily be seen that, by taking the average of these two curves, there would still be an increased rate of uptake at six hours.

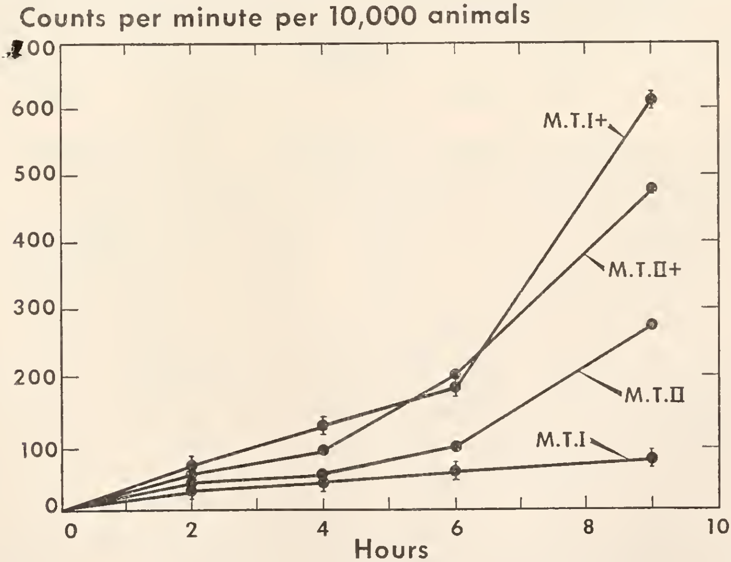

FIGURE 5. Influence of glucose on P^{32} uptake in individual. Population density, mating type I: 61,000/ml.; mating type II: 42,000/ml. Plus signs indicate cultures containing glucose.

Effect of glucose on P^{32} uptake in the individual mating types. After the usual starvation and washing procedure, glucose was added simultaneously with labeled phosphate to the distilled water into which the individual mating types were placed. This was done to determine the effect on P^{32} uptake of an added energy source as compared with the uptake resulting from the endogenous metabolism of the washed animals. The curves in Figure 5 again show the six-hour lag phase. In this instance, however, the presence of glucose was found to result in an initially greater rate of phosphate uptake, after which both mating types exhibited a sharply accentuated increased accumulation.

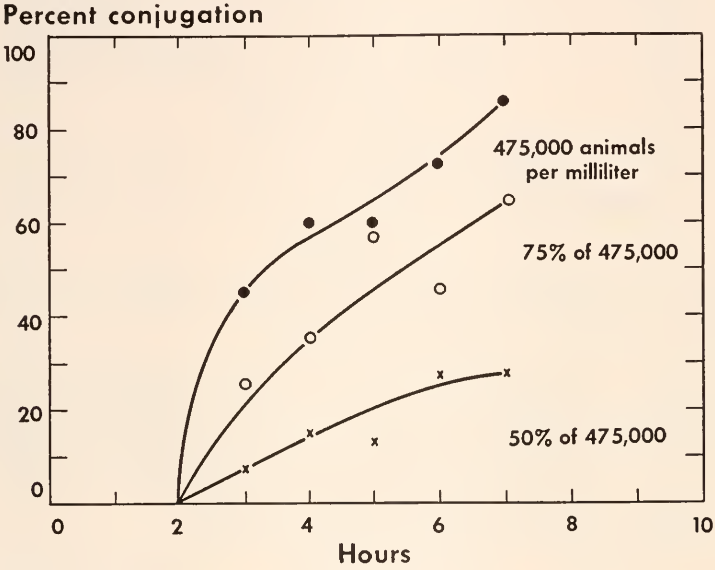

FIGURE 6. Effect of population density on rate of induction of conjugation.

Influence of population density on conjugation. It was suspected early in this investigation that the population densities used influenced the degree to which conjugation occurred. This was found to be the case, but other factors were involved, however. Cultures were found to be more fragile as they became older and since 96-hour cultures were used here in order to obtain large numbers of animals, fragmentation during centrifugation became a problem. Gentle centrifugation, just sufficient to concentrate the organisms, resulted in no fragmentation and under these conditions the subsequent percentages of conjugation were the greatest. Thus, after several hours, as much as 84% of the population were conjugating when population densities of 475,000 cells/ml. were used. Both maximal amounts of conjugation and conjugation rates within the population were found to be dependent upon the cellular densities (Fig. 6). It was found that conjugation rates were again strongly influenced by population densities and that densities higher than 260,000 cells/ml. produced considerable inhibition of conjugation.

Effect of population density on phosphate accumulation in the individual mating types. Population density (Table I) was discovered to influence the extent to which P^{32} was accumulated by either mating type. With either type, large population densities strongly inhibited ion accumulation by the individual cells. At the lower population density levels, however, mating type II accumulated more than ten times as much phosphate than did mating type I.

Accumulation of P^{32}. For comparative purposes, the number of atoms of P^{32} taken up by each mating type was calculated.

TABLE I

Influence of population density on P^{32} uptake in Tetrahymena

Population	Total CPM	Uptake $\times 10^{-4}$/cell in 9 hrs.	Uptake $\times 10^{-7}$/cell/min. CPM
3,585,000	3,788	10.6	29
1,792,500	2,002	11.2	21
896,250	1,397	15.6	29
448,125	2,572	57.4	106
224,063	11,319	505.1	935
Mating Type #2			
3,275,000	4,752	14.5	26
1,637,500	3,299	20.2	37
818,750	16,196	197.8	366
409.375	30,145	736.4	1,364

In medium containing 0.0374×10^7 atoms/volume equivalent to the average volume of one organism, mating type I was found to accumulate 7.2×10^7 atoms/organism, while mating type II accumulated 27.6×10^7 atoms/animal. When glucose was added to the medium, however, mating type II accumulated 44.1×10^7 atoms/animal while mating type I accumulated 56.0×10^7 atoms/animal.

TABLE II

Distribution of radioactive phosphorus in Tetrahymena Variety IV

Fraction	Mating Type #1 %	Mating Type #2 %
Acid-Soluble	7.6	19.5
Phospholipid	1.9	10.8
Nucleic Acid	11.7	21.6
Phosphoprotein	51.7	53.1

Intracellular distribution of phosphorus. Slightly more than one-half of the phosphorus in each mating type (Table II) was found to be localized in the phosphoprotein fraction. The nucleic acid fractions accounted for 11.7% of the phosphorus in mating type I and 21.6% in type II. Differences in the percentage of phospholipid phosphorus and acid-soluble phosphorus were the most apparent, however. Nearly five times as much phospholipid phosphorus occurred in type II as compared to that found in type I while about twice as much acid-soluble phosphorus was present in type II in comparison with that found in type I.

DISCUSSION

In their study of radiophosphate uptake in *Paramecium multimicronucleatum,* Evans and Pendleton (1952) found that feeding paramecia accumulate roughly 20 times more activity than the surrounding medium. Unfed paramecia accumulated less than one-half the radioactivity. This is in contrast to our results which show that starving *Tetrahymena* concentrates P^{32} to an extent of 450 times that present in the medium. Among protozoans, unfed amoebae have been found to concentrate more than 50 times as much P^{32} than that contained in the surrounding substrate (Mazia and Hirshfield, 1950). Evans and Pendleton (1952) found also that older paramecia cultures took up P^{32} at a more rapid rate than young cultures. This was not evident in the case of *Tetrahymena* where 48-hour cultures and 96-hour cultures appear to take up the isotope at about the same rate.

Mazia and Hirshfield (1950) found in their experiments on nucleate and enucleate cells that enucleate fragments took up considerably less P^{32} than nucleated cells. Whether the nucleus influenced phosphorus metabolism in the cytoplasm directly without handling the phosphorus itself or whether this was an indirect, long-term nuclear function is not known. In any case, we might expect a very definite nuclear effect on cytoplasmic P^{32} uptake in a process such as conjugation.

The control of conjugation is difficult. Experimental conditions must be optimum for maximum conjugation and little is known about this optimum. It is known that the mating types must first be washed free of nutrients in order to induce conjugation and that a starvation period appears to be necessary (Elliott and Hayes, 1953). Population density, however, is undoubtedly important in inducing mating in this animal.

When low concentrations of animals were mixed for the induction of conjugation, the rate of P^{32} accumulation after six hours increased 122% over the preceding phase. Nuclear stains at this time revealed the presence of the crescent stage of division in conjugating pairs. This observation at first led to the conclusion that chromosomal reduplication resulting from possible preceding DNA synthesis (Swift, 1950) was probably mainly responsible for the increased ion uptake. However, the low percentage of conjugation (maximum 30%) and probably low degree of synchrony make it unlikely that conjugation *per se* was solely responsible for the differential ion uptake. Further ion accumulation studies with separated individual mating types showed that biphasic accumulation occurred.

It is of interest to note that McDonald (1958) has demonstrated a doubling of DNA in the macronuclei of *Tetrahymena pyriformis* H. after this strain was placed in an environment which does not support growth.

When large populations (over 200,000/ml.) were mixed for mating, inducing a high percentage of conjugation with a correspondingly higher degree of synchrony, the differential uptake was not observed. The effect of numbers of organisms on ion accumulation as observed in the case of non-conjugating animals (Slater, 1957) may have complicated and obscured any biphasic accumulation here. Thus, we were faced with the problem of obtaining a high percentage of fairly synchronous conjugation wherein large populations were needed with measurable P^{32} uptake.

Since only mating type II showed the biphasic uptake of radiocative phosphorus, while type I was typified by a slow constant rate of uptake, it would seem that, in the conjugation experiments with low population densities and a correspondingly low percentage of conjugation, mating type II is probably responsible for the biphasic curve.

Of many possible explanations, the six-hour lag phase, recurring in many experiments, may represent a time of synthesis for an enzyme system to incorporate the P^{32}, or a time in which more acceptor sites for the element are created. The slow, constant uptake in mating type I might lead to the conclusion that this organism is more inactive metabolically. Under identical culture conditions, though, mating type I grows faster than type II, as indicated by colorimetric measurements (Elliott, 1949).

Significantly higher rates of P^{32} uptake were obtained in the presence of sugar. Sacks (1948) proposed that the cell membrane actively participates in phosphate transfer by the action of surface enzymes on the formation of hexose monophosphates, which by their spatial relationship to the cell surface may more easily enter. Once within, intracellular phosphatases split off the phosphorus. In this connection, Elliott and Hunter (1951) have demonstrated the presence of phosphatases in *Tetrahymena,* proposing their position to be either intracellular or at the membrane surface. Fennell and Degenhardt (1957) have located such phosphatases intracellularly in the same organism.

Notable, also, is the accentuated increase (about 215% over Phase I) in the rate of uptake of P^{32} at six hours in the types exposed to glucose. Possibly, this carbohydrate, in complementing the nutrient-free distilled water, may lead to the formation of more acceptor molecules for P^{32} than could be formed by the endogenous metabolism of the organism without the benefit of glucose. This might account for the very sharp increase of P^{32} uptake after the lag phase of synthesis.

Little difference was noted in regard to the localization of radioactive phosphorus in the phosphoprotein fractions in either mating type, but differences in the quantities of phospholipid phosphorus and acid-soluble phosphorus were quite apparent. The five-fold occurrence of phospholipid phosphorus in mating type II is probably correlated with a greater membrane permeability to this ion. The greater amount of acid-soluble phosphorus in this mating type is also probably concerned with glycolytic forces important in this process.

To evaluate the results of these experiments, various aspects of the isotope technique should be taken into consideration (Comar, 1955). In particular, possible physiological effects from the isotope-based radiation should be taken into account.

Not only should the amount of external radiation be considered, but also the degree of isotope accumulation or incorporation, which may have a greater effectiveness than otherwise anticipated. This accumulation of radioactivity may be important as a radiation hazard in *Tetrahymena* since in some of these experiments the organism accumulated large amounts of the isotope. Radiation effects were probably minimal, though, because of the short time intervals employed. Also, at the isotope concentrations used ($0.1\ \mu c.$–$0.2\ \mu c.$) no overt effects were noticed when compared to the controls. Since *Tetrahymena* does not appear to be noticeably influenced by radiation dosages less than about 5×10^5 roentgens (Elliott and Slater, 1951), it is unlikely that the isotope levels used were significant. Imperceptible nuclear or membrane changes may, of course, have occurred, though.

The isotope effect resulting in a preferential rate of incorporation of isotopes differing in mass and mobility into biological systems may also be considered here.

These effects may be of significance only in experiments involving diffusion-like processes based on organic reactions, but even then the magnitude of the effect is usually less than 5% and may pass undetected in most biological experiments. Since P^{32} and P^{31} are essentially alike in their biological reactions the isotope effect can probably be neglected here.

The type of radiation of an isotope is important especially in regard to the counting instrumentation used. The scintillation detector used, though more adapted for gamma counting than for beta rays, was sensitive enough to register the hard beta rays in a reasonably efficient manner. Concentrated suspensions of *Tetrahymena* plus P^{32} yielded counts essentially the same as similar volumes of water plus the isotope.

As commented on before, care was taken to keep the geometry of the samples to be counted as constant as possible. This was especially necessary because of the extreme sensitivity of the scintillation detector to low-energy, scattered radiation.

In order to demonstrate a significant relationship between P^{32} uptake and chromosomal reduplication by the method we have chosen, as was an original intent of this study, many animals would have to be brought to nuclear stages as synchronously as possible.

Although almost 100% conjugation with good synchrony can sometimes be obtained in depression slides (Elliott and Hayes, 1953) the volumes used are too small for our methods. Zeuthen's (1954) methods for the induction of synchronous mitosis in *Tetrahymena* by means of alternate thermal shocks might prove useful for producing near-identical nuclear conditions in the production of synchronous conjugation, but thus far this synchronous mitosis has been induced only in amicronucleate strains (Zeuthen, 1956, private communication). It also seems apparent that the individual strains behave quite differently when treated in this manner, with a lesser degree of synchrony obtainable with strain E., for instance.

Constant conditions of centrifugation were employed to minimize cell breakage or stress during harvesting and washing, since Evans and Pendleton (1952) have demonstrated a leakage of P^{32} from paramecia washed by centrifugation. This loss did not seem to occur with *Tetrahymena*. Experiments designed to discover such leakage showed that P^{32} was completely retained over an eight-hour period.

SUMMARY

1. *Tetrahymena* in distilled water containing P^{32} concentrated the isotope to an extent of 450 times that contained in comparable volumes of water after nine hours.

2. The accumulation of P^{32} by individual *Tetrahymena* varied with population density. With large populations, the amount of phosphate accumulated per cell was sharply reduced, whereas with low population densities larger quantities were accumulated. More than ten times as much P^{32} was found in mating type II than in mating type I.

3. The induction of conjugation was found to be strongly influenced by the amount of cellular debris present. The greatest amount of conjugation was obtained by the use of large population densities with gentle centrifugation. It was suspected also that age as influencing cell fragility was important here.

4. Mating type II showed a significantly increased rate of P^{32} accumulation six hours after addition of this isotope in distilled water, while mating type I was found to concentrate P^{32} at a slow constant rate.

5. Three times as much phosphate was accumulated during nine hours in the presence of glucose as when in distilled water alone with either mating type.

6. Although a biphasic accumulation was noted with low population densities prepared for the induction of conjugation, this was probably primarily because of an increased uptake by mating type II. Similar information was not obtained with large population densities, possibly because of the numbers effect.

7. Nearly five times as much phospholipid phosphorus was found in mating type II as compared to mating type I. This appears to be correlated with the greater ion accumulation ability found in type II. Only about twice as much acid-soluble phosphorus was found in type II compared with type I, however.

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IONIC AND WATER BALANCE OF PLANARIANS[1]

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Planarians, order Tricladida, class Turbellaria, are flatworms, mostly free-living, that have invaded fresh water, the oceans and land. All have the same general morphology and, except for some of the land planarians, are relatively small, usually not exceeding a centimeter or so in length. All are unarmored and most of them are ciliated. All have a flattened, leaflike body and a much branched intestine with a single external orifice (Hyman, 1951).

Little is known about the mechanisms whereby these organisms can adjust and maintain water and ionic content in such varying habitats. Information that is available is, except for a small amount of very recent work, summarized excellently by Hyman (1951). While some variations in the excretory system have been noted between marine and fresh-water forms, most of our physiological knowledge consists of demonstrations that planarians are influenced by their osmotic environment, swelling or shrinking appropriately as the medium is changed (Adolph, 1925). Some planarians are euryhaline and one (*Gunda* = *Procerodes*) has been studied by Pantin (1931) and by Beadle (*cf.* Beadle, 1934). A discussion of this work is to be found in Krogh's book (Krogh, 1939). The present report gives certain basic information about water and ionic contents of marine and fresh-water planaria, together with preliminary studies of fluxes of the materials into or out of the animal body. The animals were chosen, in large part, because of their availability. The common laboratory planarian, *Dugesia tigrinum,* was used as the fresh-water form, the ectoparasite of *Limulus, Bdelloura* (species not determined) for the marine animal. Regrettably, *Gunda* was not available at the time these studies were carried out.

MATERIALS AND METHODS

Dugesia are maintained in pond water stock culture in this laboratory. They are fed frequently on raw liver and are fine, healthy animals relapsing now and then into a sexual phase. The cultures are usually propagated by cutting the animals in half transversely and then allowing regeneration to take place.

Bdelloura were obtained from *Limulus* collected on Cape Cod. The worms are easy to obtain and live well in sea water. While closely resembling *Dugesia* in general internal structure, size and shape, behavior is quite different. Compared to *Dugesia* they are very active, looping along with quiet vigor by means of the adhesive organs. *Dugesia*-like gliding is rarely seen. So far, attempts to feed *Bdelloura* have failed. Similarly, attempts to demonstrate chemical attraction to various portions of *Limulus* have failed in spite of the fact that *Bdelloura* is reported to exist only on *Limulus*.

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Both types of worms were transferred, as desired, by pipettes or small spatulae. To obtain wet weights, animals were drained on fine fibered filter paper and then transferred to tared strips of Parafilm for rapid weighing on a torsion balance.

Na and K were determined by flame photometry on extracts prepared by heating worms (20–60-mg. samples) in 10 ml. of H_2O with 3 to 4 drops of glacial acetic acid added. Nitric acid digests of residues from the hot acid water extraction showed that essentially complete removal of the ions had taken place. Chlorides were determined on the same extracts by means of a Cotlove type chloride meter.

Worms were labeled with Na^{24}, K^{42} and Cl^{36} by standard methods.

In general, influxes of the ions were determined by counting radioactivity in individual worms placed on a waxed plate directly under a thin-window Geiger counter tube. Levels of activity were chosen such that one-minute counts would suffice, the same worm being assayed at appropriate intervals. Effluxes of the ions were determined by essentially the same procedure, zero time being the time of immersion of the animals in the non-radioactive solution (10^2 to 10^3 times the volume of the worm) following a labelling period in radioactive solution. Tritiated water efflux was determined on worms labelled overnight in tritiated water by transferring them rapidly with stirring to 1-ml. volumes of non-tritiated medium. Aliquots of the washing media were then counted in the liquid scintillator. "Inulin spaces" were determined by adding C^{14}-labeled inulin in a few instances, dry aliquots of extracts being counted in a gas flow counter.

TABLE I

Summary of data for Dugesia and Bdelloura taken from normal cultures or sea water. Concentrations as mM/Kg. Inulin values determined by C^{14} *labelled inulin in trace amounts, calculated as* $\frac{\text{counts/Kg animal}}{\text{counts/Kg medium}}$. *Numbers in parentheses indicate number of determinations averaged.*

	Na	Cl	K	Inulin value	% Dry
Dugesia (22)	14.6 ± 0.5	18.8 ± 0.7	38.2 ± 0.7	2.0	22
Normal *Dugesia* medium*	0.2	0.3	0.02		
Dugesia, (2) (in high salt)	20	28	27		
Bdelloura (20)	122 ± 1.4	135 ± 2.1	97 ± 0.3	5.7	25
Sea water	440	510	10		

* Concentration varied, in general between limits for Na, 0.8-0.1 m*M*. High salt medium for Dugesia = NaCl 10 m*M*, KCl 10 m*M* in pond water.

RESULTS

Ionic and water contents

Table I summarizes the analytical data for both worms. As is usual in comparing marine and fresh-water forms, Na and Cl concentrations are quite different, whereas potassium concentration varies less. *Dugesia* concentrates all three ions, *Bdelloura* concentrates K but maintains low concentrations of Na and Cl as compared to the environment.

Some attempts have been made to acclimatize *Dugesia* to high salt concentrations with little success as yet. As soon as the salt concentration of the medium begins to approach that of the animals, they appear very unhappy and stop feeding. On the other hand, worms kept for two weeks in a 20 m*M* mixture of NaCl and KCl in pond water, during which time they did not feed, recovered and enjoyed a good liver meal within 24 hours after being placed back into normal pond water medium. Table I includes analytical data on the average of two samples of animals from the high salt culture. K decreases by about 30% as compared to controls in spite of a 500-fold increase in external K. Na and Cl contents of the animals increase nearly 50%, the external concentrations having gone up about 100 times. Clearly the internal ionic composition of the worms is not highly dependent on the ionic composition of the medium. A variety of studies has been made on starved worms, worms kept at low temperature, worms cut and allowed to regenerate and even worms maintained in distilled water for several hours. The results show a most remarkable ability of *Dugesia* to maintain constant internal water and salt concentrations under all these conditions.

TABLE II

Final relative weights and ionic contents of Bdelloura maintained at least two days in sea water diluted as indicated. A series, average of four analyses, B series, average of two analyses. Concentrations as mM/Kg final wet weight or mM/liter of medium.

		Na_i	$\frac{Na_i}{Na_o}$	Cl_i	$\frac{Cl_i}{Cl_o}$	K_i	$\frac{K_i}{K_o}$	Relative weight
100% sea water		134	0.30	135	0.27	97	9.7	100
75% sea water	A	98	0.30	108	0.28	87	11.6	114
	B	75	0.23	96	0.24	80	8.0	118
50% sea water	A	52	0.24	62	0.25	65	12.0	151
	B	56	0.25	67	0.27	64	6.4	165
40% sea water	A	51	0.29	61	0.20	52	13.0	189
	B	61	0.34	61	0.28	52	5.2	210
30% sea water	A	30	0.23	36	0.23	45	15.0	213
	B	35	0.26	51	0.31	45	4.5	221

Murray (1927), in a classic study, notes that a medium of osmolarity of about 15 m*M* NaCl is optimum for growth of *Dugesia* explants in tissue culture.

Bdelloura can live for two weeks or more in diluted sea water. In concentrations less than 50% sea water, however, the worms move only sluggishly and look as though they are in poor condition. In dilute sea water, the worms increase in body weight and show no signs of return to original size while in the experimental medium. Normal weight is regained on return to normal sea water. Table II gives data on weight changes and body concentrations of Na, Cl and K in worms held two or more days in sea water diluted either with distilled water (A) or 10 m*M* KCl (B) in order to hold the K level of the environment approximately constant. Na and Cl concentrations of the worms adjust to the various dilutions as indicated by relatively constant inside-to-outside ratios. K concentrations of the animals decrease in proportion to the increase in body weight due to swelling in dilute media, tending to maintain a constant amount of K per gram original weight, regardless of the external concentration of K or of total salt.

Flux rates of ions and water

With the use of radioactive tracers, K^{42}, Na^{24}, Cl^{36} and tritiated water, flux rates have been determined for *Dugesia* and *Bdelloura.* These results are reported in preliminary form since the detailed treatment would require a considerably greater number of observations and, hopefully, a more detailed understanding of the physiological mechanisms involved.

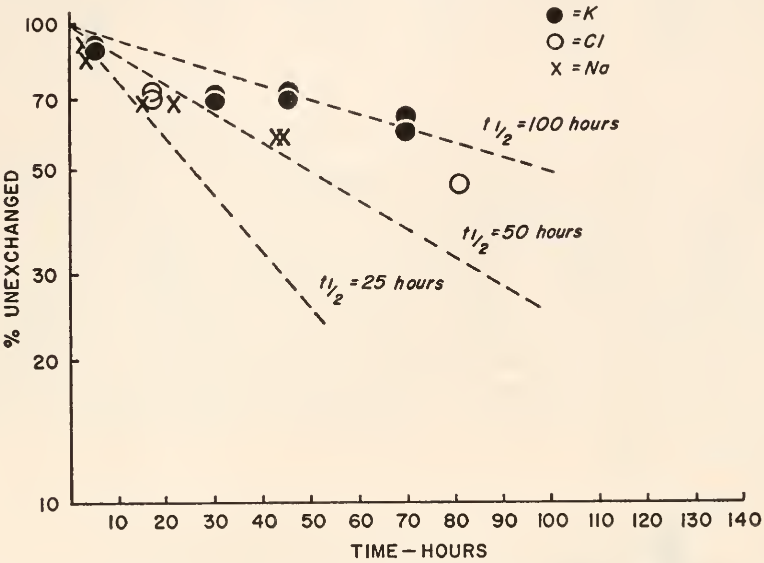

FIGURE 1. Influx of K^{42} (circles), Na^{24} (dots) and Cl^{36} (crosses), added in trace amounts to normal culture medium, into *Dugesia.* Results plotted as % unexchanged ion of the worms against time of immersion in radioactive solution. Straight lines, representing different $t_{\frac{1}{2}}$ values as indicated, are drawn merely to show the variations in slopes of lines, not to fit the experimentally determined values.

Figures 1, 2 and 3 give results for ion fluxes, on semi log paper, as % unexchanged ion in the worm plotted against time.

Influx of ions into *Dugesia* is very slow following an initial rapid entry (Fig. 1) of unknown significance. The initial rapid phase amounts to over 10% whereas inulin spaces are much less. Therefore, more than the "drainage compartment" is involved. The slow influx, roughly exponential with time, has a $t_{\frac{1}{2}}$ value of over 100 hours for all ions tested. Because of this very slow labelling, very few efflux rates were determined. Scattered results, however, indicated that efflux was at least as slow as influx for each ion.

Raising the external K content did increase influx of K^{42} (Table III). How-

TABLE III

Per cent specific activity of K^{42} in Dugesia after equilibration with K^{42} solutions of ionic composition indicated for five hours. Concentrations in mM/Kg. Concentrations measured for solution # 1, calculated for the others. The worms in solutions 3 and 4 showed definite signs of deterioration.

	Medium, m*M*			Planaria			K^{42} Spec. Act.
	Na	Cl	K	Na	Cl	K	
1.	2.4	3.0	1.0	13.3	17	37	6.5
2.	12.0	23.0	11.0	16.4	20	39	8.3
3.	22.0	33.0	21.0	18.7	23	41	13.0
4.	52.0	103.0	51.0	20.4	31	51	21.0

ever, the increase in influx was slight as compared to the external concentration change.

Influx of Na^{24} into *Bdelloura* is complicated (Fig. 2). There appears to be an "unexchangeable" portion of Na in the worms but results are variable with respect to this phenomenon and further investigations are needed.

K^{42} influx into *Bdelloura,* following the usual initial rapid phase, seems simple and uncomplicated, the $t_{\frac{1}{2}}$ value being about 3% of that for *Dugesia.* Since *Bdelloura* and *Dugesia* are similar in size and shape, $1/t_{\frac{1}{2}}$ values for influx, divided by the respective external concentrations, could give a measure of relative permeabilities. In view of the data in Table III, this procedure is questionable but emphasizes that the marine form is considerably more permeable to ions than is the fresh-water form.

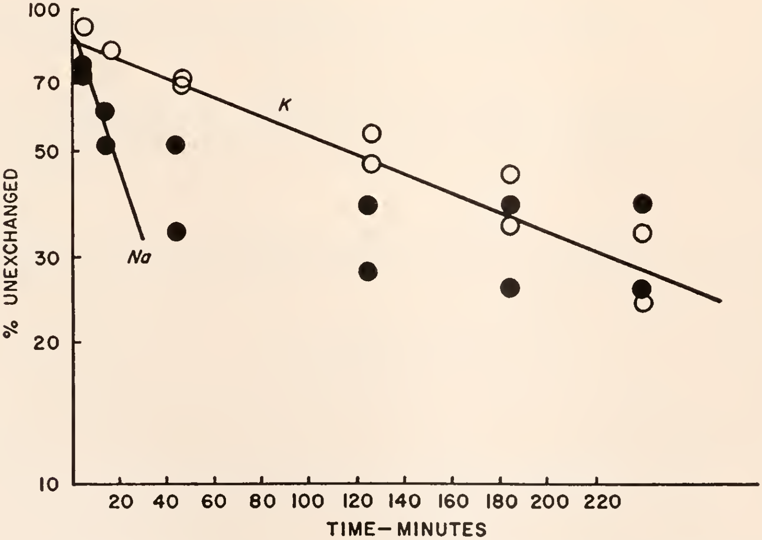

FIGURE 2. Influx of Na^{24} (dots) and K^{42} (circles) into *Bdelloura.* Radioactive element added in trace amounts to normal sea water. Results plotted as in Figure 1.

Effluxes of Na^{24} and K^{42} from previously labelled *Bdelloura* are shown in Figure 3. $T_{\frac{1}{2}}$ values for efflux are essentially comparable to those for influx of the respective ions. As with the influx measurements, K^{42} movement seems relatively uncomplicated, there are again indications of an inexchangeable, or very slowly exchangeable Na fraction.

Tritiated water exchanges very rapidly. The two cases graphed (Fig. 4) represent the smallest (10 mg.) and the largest (55 mg.) *Bdelloura* used. Since body surface is related to weight (see later discussion) it is not surprising that rates of water outflux, measured as half-times of exchange, divided by weights give nearly identical values. For animals of comparable size, efflux rates into 50% sea water following labelling in 50% sea water are identical with those into normal sea water.

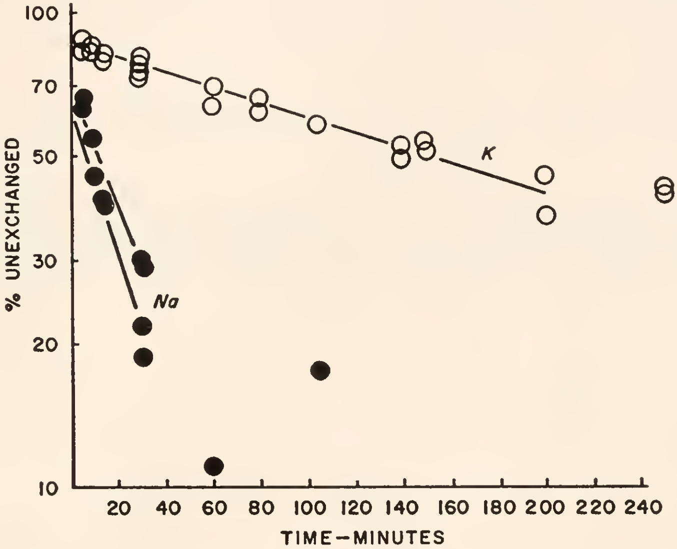

FIGURE 3. Efflux of Na^{24} (dots) and K^{42} (circles) from *Bdelloura* to normal sea water. Worms labelled overnight in sea water with trace concentrations of radioactive ions. Results plotted as in Figure 1.

Similar measurements on influx of tritiated water into *Dugesia* yielded $t_{\frac{1}{2}}$ values of about 160 seconds. Since these animals weighed around 10 mg. the results indicate a somewhat slower rate of water movement in the fresh-water animal.

It should be noted that the $t_{\frac{1}{2}}$ values read from curves such as those of Figure 4 give information on flux rates and are not to be treated as indicators of permeability except to show a very rapid exchange and probably a real or potential rapid net change. Nonetheless both marine and fresh-water forms can be characterized as freely permeable to water and there is certainly no indication that adaption to fresh-water existence has involved the development of a high resistance to the passage of water.

DISCUSSION

The pattern of electrolyte distribution in the two flatworms studied has some interesting general features illustrated by Figure 5. The data on ionic contents

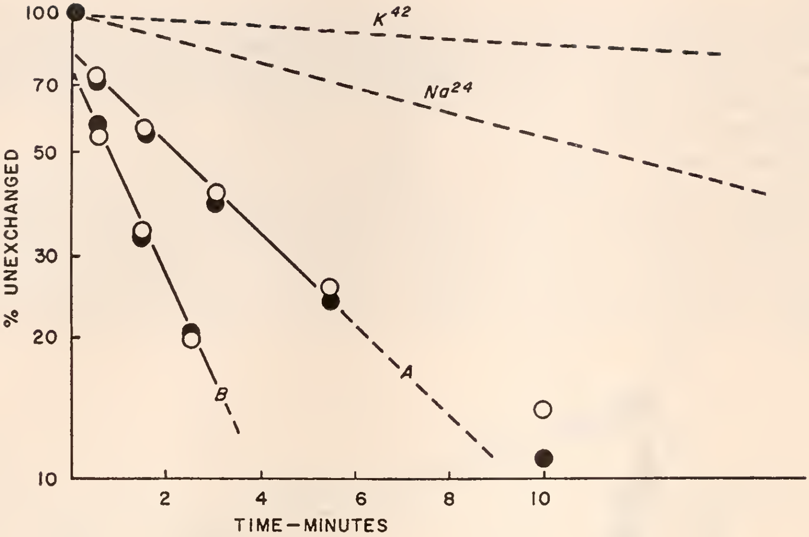

FIGURE 4. Efflux of tritiated water from *Bdelloura*. Animals were equilibrated overnight in tritiated sea water. Curve A, animals in the 55-mg. weight range. Dots: entire procedure in normal sea water, circles: in 50% sea water. Curve B, same as curve A except animals in 10-mg. weight range. K and Na lines from Figure 3.

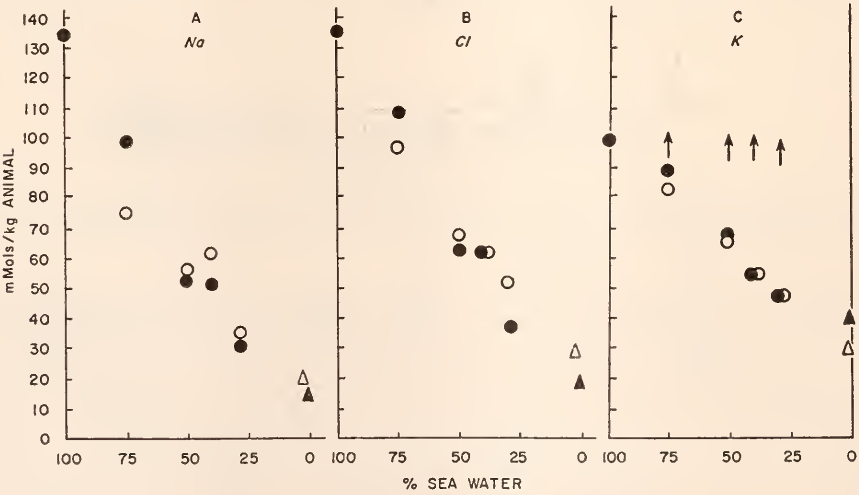

FIGURE 5. Ionic concentrations in *Bdelloura* in different concentrations of sea water as shown in Table II. Concentrations in animals as m*M*/Kg. final wet weight; in sea water as % sea water. Dilutions of sea water either with distilled water (dots) or 10 m*M* KCl (circles). In C, arrows point to K concentrations in animals calculated on basis of original, rather than final wet weights. Triangles represent ionic concentrations in *Dugesia* from Table I. Solid symbols, animals in normal medium; open symbols, animals in high salt medium.

for *Bdelloura* given in Table II are plotted against the relative concentration of sea water. The Na, Cl, and K contents of *Dugesia* in normal and high salt media (Table I) have been added to the figure. As previously noted, *Bdelloura* in dilute sea water swell to a new volume which is then maintained, thus indicating that they do not acclimatize in the sense of adjusting their weights (and presumably internal concentrations) back towards normal. The increase in body volume is such that, while K concentration decreases, the total amount of K per animal does not change. Inspection of Figure 5c shows that, in a sense, *Dugesia* is a dilute marine form with respect to K. Na and Cl contents of *Bdelloura* decrease more rapidly than K, the internal concentrations being an almost constant fraction of the external. *Dugesia,* however, concentrate both ions in excess over the environment. It is possible that the sodium extrusion mechanisms of the marine form are converted to, or displaced by, a sodium uptake mechanism in *Dugesia*.

TABLE IV

Flux rates of Na, Cl, K and THO (or H_2O) estimated from data in this paper and from other sources as indicated. The values should be taken to indicate order of magnitude only. Influx values unless otherwise noted.

	pM/cm.²/sec.			ml./cm.²/sec. ×10⁻⁵
	Na	Cl	K	H_2O
Bdelloura. Efflux to sea water	14.5		1.26	11
Dugesia. (pond water)	0.006	0.006	0.015	<11
Frog.[a] (pond water)	13.8			1
Isolated frog skin.[b] (Ringers)	280			11
Erythrocytes.[c] (Plasma)	0.15		0.026	570
Ascites cells.[d] (Plasma)	3.0		3.0	
Halycystis[e]	10.0	426		
Frog Muscle.[f] (Ringers)	4		4.1	

[a] Jorgensen, 1949.
[b] Ussing, 1955.
[c] Solomon, 1952, and Paganelli and Solomon, 1957.
[d] Hempling, 1958.
[e] Blount and Levedahl, 1960.
[f] Mullins, 1959.

In his pioneering studies of *Gunda,* using conductivity methods, Pantin (1931) found that normal animals in sea water had an internal electrolyte concentration equal to about 0.3 *M* NaCl; animals acclimatized to tap water, a concentration of 0.036 *M*. It is, of course, impossible to assign specific ionic values but the general change is in the same range indicated by the comparison of *Dugesia* and *Bdelloura* in Figure 5.

At present it would be premature to calculate any precise values for cellular concentrations. Inulin spaces are so small that they almost certainly indicate only non-draining external fluids rather than intra-animal extracellular spaces. Histological sections of the worms are of little help, except to show that the mesenchyme regions are indeed loosely organized. The points for Na or Cl of Figure 5 lie near a line whose gradient could be interpreted as indicating a "space" of about 25%. This, in turn, would yield a "non-chloride space" K concentration for *Bdelloura* of 130 m*M* and for *Dugesia* of 51 m*M*.

Table IV gives some calculated flux values for *Bdelloura* and *Dugesia,* together with representative values for other forms. Calculation of flux rates has been on the basis of half time exchange values, as illustrated in Figures 1–4, and estimates of body surface. Body surface areas for both types of worms were estimated by tracing outlines of the animals on graph paper, cutting out and weighing the tracings and comparing them with weights of standard areas of the same paper stock. Especially in the case of *Bdelloura,* these values must be regarded as approximate. In normal resting state, *Bdelloura* body surface obviously is not smooth. Both worms can be greatly flattened without losing weight. Hence the surface must be extensible. Even casual inspection of active worms indicates changes in ratios of length/width, width/thickness and so forth. For worms (*Dugesia* and *Bdelloura*) in the weight range of 7–30 mg., the points for surface areas, as derived from weights of paper outlines, plotted against weight of worms, fell fairly well along a straight line indicating 3.5 mm.^2 for each mg. body weight of worm (see Clark and Cowey, 1958, for a discussion of weight-surface relationship in flatworms). This figure has been used in calculating the flux rates for both worms.

No marked differences between influx and outflux values were noted for either worm nor, with *Bdelloura,* was there much effect on either influx or outflux of Na, K or THO of changing the external medium from sea water to 50% sea water. With *Dugesia,* increasing the external K concentration did increase the rate of K^{42} entrance but the increase was small compared to the increase in external concentration. The general implication thus is that the movement of the ions into and out of the flatworm is dependent on the properties of the worm and not on the magnitude of a diffusion gradient. The ions measured do not appear to be diffusing along free pathways.

Ions pass in and out at a greater rate in the marine worms than in the fresh-water *Dugesia.* Even if approximate permeability constants are calculated, by dividing flux rates by concentrations, the marine forms are ten or more times more permeable to the ions measured. In *Bdelloura,* sodium influx is higher than potassium influx. In this case, however, conversion of flux rates to relative permeabilities (14.5/440 for Na and 1.26/10 for K) shows the customary lower "permeability" for Na than for K. However, as noted, influx of Na^{24} is about the same from either 50% sea water or normal sea water.

In colonizing the marine and fresh-water environments, the flatworms have been faced with severe osmotic and ionic problems. Fresh-water forms are more dilute, have developed mechanisms for uptake of Na and Cl in addition to a K-concentrating system and must possess a most remarkable system for removing excess water. The results presented here certainly do not indicate the development of an impermeability to water nor, in the light of the low ionic flux rates, is it likely that a filtration resorption system in an excretory device will be found to be the mechanism used. The salt water flatworm may be assumed to be in water equilibrium with its environment, "deficits" due to low ionic contents being made up by organic solutes. On the other hand, *Bdelloura* appears reasonably permeable to ions and yet maintains low body Na and Cl and high K as compared to the environment. Thus a major problem of the salt water form must be the extrusion of cations, a problem found in all cells of higher animals and of marine forms studied so far.

There is, unfortunately, no evidence to show whether ion uptake by fresh-water forms is mediated by the same system, working in reverse, that accounts for ion extrusion in marine forms. Similarly, one cannot tell whether ion movements are linked to water movements, especially in the fresh-water forms.

SUMMARY

1. The Na, K and Cl concentrations (mM/Kg. wet weight) of *Dugesia tigrinum* are 14.6, 38.2 and 18.8. For the marine triclad *Bdelloura* the concentrations are, respectively, 122, 97 and 135.

2. Flux rates for the ions and for tritiated water are faster in the marine flatworms. Exchange of tritiated water, however, is very fast (half time values < 3 minutes) for both forms.

3. *Bdelloura* can live for extended periods of time in dilute sea water, swelling to a new volume which is maintained until returned to a normal sea water medium. *Bdelloura* swollen in dilute sea water do not lose K, even though the K concentration drops due to the volume increase.

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STUDIES ON VISCERAL REGENERATION IN SEA-STARS. I. REGENERATION OF PYLORIC CAECA IN HENRICIA LEVIUSCULA (STIMPSON)[1]

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The ability to regenerate lost parts is well developed, and well known, in sea-stars. Some forms, indeed, such as *Linckia* and *Coscinasterias,* regularly reproduce by spontaneous autotomy or fission followed by extensive regeneration of all missing portions. In general, however, asteroids utilize their regenerative capacity only to the extent of replacing relatively minor parts of the body, rays or portions of rays, lost as a result of accident or through autotomy following restraint or injury. The regenerative process is so widespread and conspicuous that it has naturally been extensively studied. Hyman (1955, p. 314) summarizes the results of several earlier investigators in the following statement: "In all species tested arms cut off at any level are readily regenerated, although the process is relatively slow. Regeneration takes place in the same manner as postlarval growth; the tip of the arm, marked by the terminal plate and tentacle and the optic cushion, appears first, and other structures are then formed between this tip and the stump in a central direction with the youngest structures just proximal to the new tip. The pyloric caeca are replaced by outgrowth from the old ones and similarly the radial water canal and radial nerve by outgrowth from the stumps of these structures."

It seems obvious that if, as has been established, the sea-star can regenerate pyloric caeca in the course of replacing an entire autotomized ray, the animal should easily be capable of regenerating them when only these organs have been operatively removed from an otherwise intact ray. Experiments to demonstrate this point have apparently not been reported, however, although studies on regenerating caeca under these conditions could be directed towards answering a group of very interesting questions related to the details of caecal regeneration. For example, it would be of interest to locate the growing point of the regenerate; is it just behind the advancing tip, as in the parietal components of a regenerating ray, or is it at the point of outgrowth from the cut stump? Hyman's statement suggests a fundamental difference between the mode of regenerative growth in the body wall and that characteristic of the caeca, radial nerve, and radial water

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vessel. Another question involves the possible role of the suspending mesenteries in guiding the growth of the regenerating caeca; it will be recalled that Kille's studies on gut regeneration in *Thyone* (1935) and Dawbin's account of the corresponding process in *Stichopus* (1949) demonstrated an intimate involvement of the mesentery-edge in early regenerative stages, and the torn mesenteric remnants may function similarly in asteroids. In addition, the presumably undifferentiated cells making up the early regenerate must at some point become transformed into the several highly specialized types of cells characteristic of the normal pyloric caecum, and the details of such transformation would be of interest. Finally, in such sea-stars as *Henricia* and its relatives the pyloric caeca are not so simple as in the asteriids but are accompanied by elaborate flagellary pumping organs, the so-called Tiedemann's pouches, which like the caeca develop as radial branches of the pyloric stomach. If the regenerating caecum "grows out" from its cut stump, does the pouch also advance from its own stump, or is it elaborated later by differentiation from the overlying caecum?

Answers to questions such as these, and the details of the regenerative processes, were sought in a series of experiments involving operative removal of the pyloric caeca in several species of sea-stars. The results to be reported here are those obtained in a somewhat limited number of operations performed on *Henricia leviuscula,* following which the experimental animals were observed for a maximum of eight weeks. This series, though small and of brief duration, is sufficient to provide basic information on several of the general questions involved, and discussion of these results will provide a background for subsequent accounts dealing with other species and other portions of the digestive system.

These studies were begun during tenure of a John Simon Guggenheim Memorial Fellowship in 1958–59, at the Hopkins Marine Station of Stanford University, Pacific Grove, Calif. The generosity of the Guggenheim Memorial Foundation, and the hospitality and assistance of the Director and staff of the Hopkins Marine Station, are gratefully acknowledged.

Material and Methods

Small to moderate-sized specimens of *Henricia leviuscula* (radius averaging about 2 cm.) were collected beneath stones in the intertidal area at Point Piños, near Pacific Grove, Calif. Stocks of these animals were maintained in running sea-water in one-gallon jars fitted with escape-proof collars of plastic screening. Although offered a variety of food, the specimens were never observed to feed; they remained in apparently vigorous condition, however, for periods of two months or more. The suggestion has been made elsewhere (Anderson, 1960) that *Henricia* is capable of subsisting on suspended particulate matter.

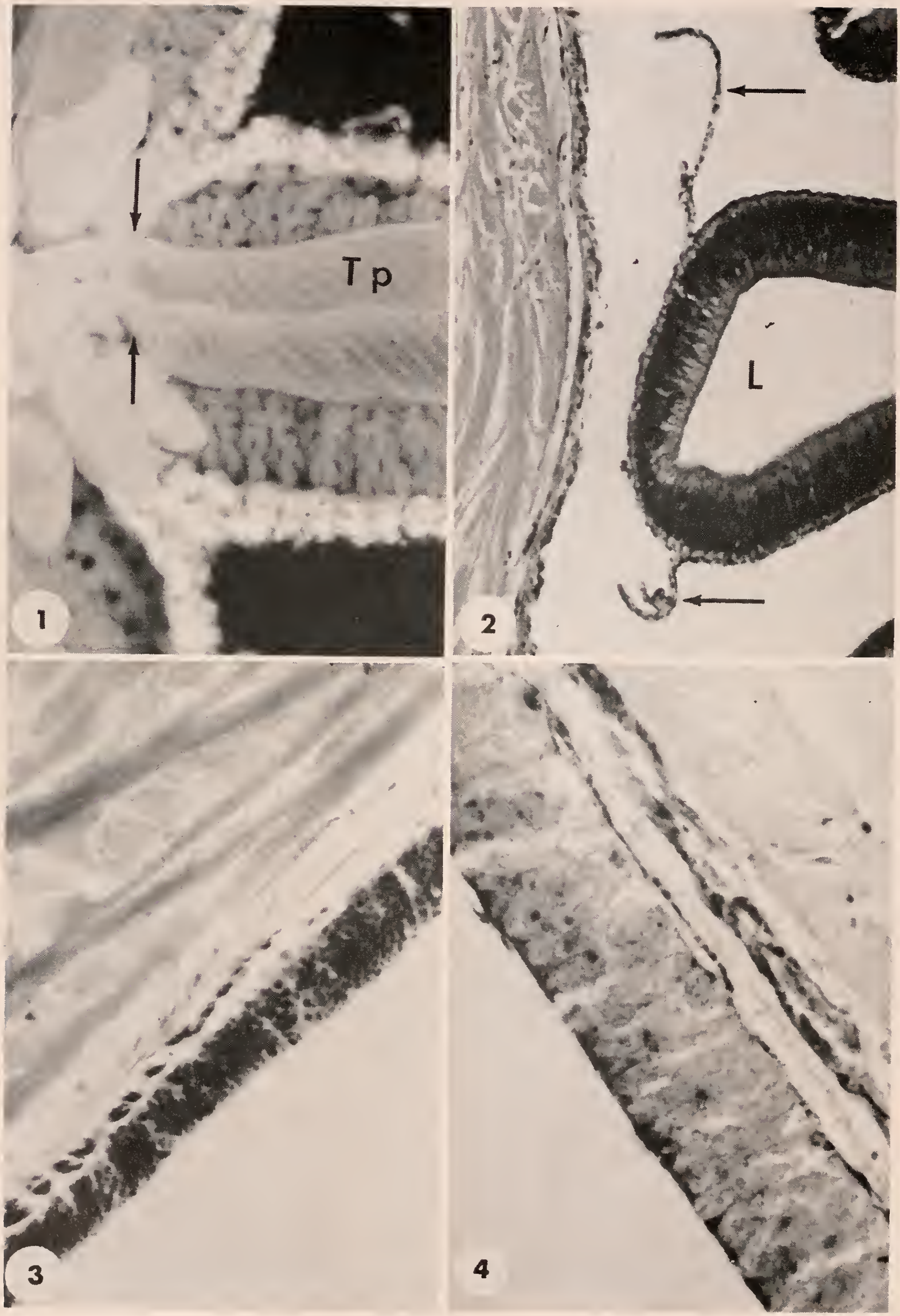
Selected specimens were immobilized by soaking for 15 minutes in $MgCl_2$ solution (8% in tap water). Using iridectomy scissors, a median longitudinal incision was made through the aboral body wall of one ray. Through this incision the paired pyloric caeca of the ray, together with their associated Tiedemann's pouches, were removed by grasping the caeca in watchmaker's forceps and either tearing or cutting their mesenteric attachments inside the aboral body wall, finally transecting the pyloric ducts and Tiedemann's pouches as near as possible to their proximal origins from the pyloric stomach (Fig. 1). Identification of individual specimens was facilitated by performing the operation on different rays, numbered consecutively from the madreporite, by clipping off the distal tips of different combinations of rays, and by noting distinctive features of the highly variable color patterns of different individuals.

Returned to running sea-water, the animals recovered rapidly from the effects of the magnesium treatment and of the operation. Without suturing or other assistance the edges of the incision gradually approached each other, beginning proximally, and one week after the operation the wound was usually closed by a thin and rather delicate white web of connective tissue. By the end of the second postoperative week healing was well advanced, and the site of the incision was recognizable only by a persistent furrow in the body wall, by its somewhat lighter color, and by the absence of papulae and spines. These differences gradually became less conspicuous during following weeks, but the furrow was still noticeable after four weeks.

Internal events could be checked only by sacrificing the animal. At weekly intervals, selected individuals were again soaked in $MgCl_2$ solution to prevent movement, and all rays except the operated one cut off near the disk. By making an "equatorial" incision through the body wall all around the animal, and cutting the digestive tract across at the junction between cardiac and pyloric stomachs, oral and aboral portions of the body were separated. The aboral portion, bearing the pyloric stomach and any regenerated parts of the caeca in the operated ray, was inverted and pinned out flat, face up, in a small wax-bottomed dissecting pan, using fine glass needles. After gross examination, the preparation was sketched or photographed, flooded with Helly's fluid, covered, and allowed to fix and harden for several hours. At the end of this preliminary fixation period, the specimen was trimmed and the significant portions transferred to a vial of Helly's fluid for an additional 24 hours' fixation. The tissue was then washed overnight in running water and placed in a decalcifying solution (disodium EDTA, 5% aqueous) where it remained for one week. The specimen now consisted of the delicate regenerating parts of the caeca, attached to and supported by the tough, decalcified adjacent portion of the aboral body wall. This was dehydrated and embedded in paraffin by standard technics and 7 μ serial cross-sections prepared. The sections were stained with Mallory's phosphotungstic acid hematoxylin (PTAH), Harris' hematoxylin and fast green, or a periodic-acid-Schiff (PAS) routine followed by fast green and Weigert's acid-iron-chloride hematoxylin. The PTAH technic was particularly useful in demonstrating flagellary basal bodies and intracellular secretory granules, as well as in distinguishing between muscle and collagenous tissue; the PAS routine revealed membranes and mucous gland cells.

RESULTS

The experiments and observations, which followed the changes in operated animals for a maximum of only 8 weeks, clearly demonstrated that even in this relatively brief period some progress is made toward regenerative replacement of extirpated pyloric caeca. The process of regeneration involves an orderly, consistent series of events which may now be explored and described; before



FIGURES 1–4.

proceeding to this, however, it will perhaps be helpful to review briefly the normal anatomy and relationships of the pyloric caeca and adjacent structures.

The body wall is covered internally by the parietal peritoneum, a generally flattened, cuboidal, flagellated epithelium in which only occasional cells contain accumulations of granules staining intensely with PTAH. Along two parallel lines above each pyloric caecum this layer rises to form the paired mesenteries by which the caecum is suspended from the aboral body wall. Although these mesenteries consist fundamentally of the standard two mesothelial layers separated by mesenchyme, it is noteworthy that in *Henricia* they are not continuous sheets but form a highly fenestrated, weblike tissue (Fig. 2). It is these suspensory webs that were cut or torn apart in the operative removal of the pyloric caeca. The mesothelium of the mesenteries is continuous with the visceral peritoneum, which forms the outermost layer clothing the pyloric caecum and its appended Tiedemann's pouch. Over these organs the mesothelium is markedly flattened, and the nuclei of its flagellated cells are peculiarly bean-shaped. Figure 2 shows the relationships between the median duct of a caecum, the paired mesenteric webs, the parietal peritoneum, and the overlying aboral body wall. Further anatomical and histological details of the pyloric caeca need not be described here; characteristics of the tissues involved and the distribution of individual cell types have recently been elucidated (Anderson, 1960). It should perhaps be noted, however, that the epithelium of the median caecal duct contains an extremely high concentration of zymogen cells packed with secretion droplets demonstrable by the PTAH technic; this characteristic is of significance as a recognition feature for remnants of the duct in regenerating tissues.

Following operative removal of the pyloric caeca, the first week is marked only by early stages in the healing of the incision, with no noticeable internal changes. At the close of the second postoperative week, however, although there are no conspicuous signs of caecal regeneration, sections reveal that preliminary events are in progress. One of these consists of a very marked hypertrophy of the parietal peritoneum; its normally flattened, cuboidal cells have now become several times taller than usual and are packed with coarse spherules (Fig. 3). These cytoplasmic inclusions are PAS-negative (Fig. 4), and in sections stained with Harris' hematoxylin and fast green they are neither basophilic nor markedly acidophilic; they do, however, stain intensely blue-black with PTAH. In staining behavior, as in shape and size, the spherules resemble those occasionally found in scattered peritoneal cells in the normal animal, as well as the abundant secretory

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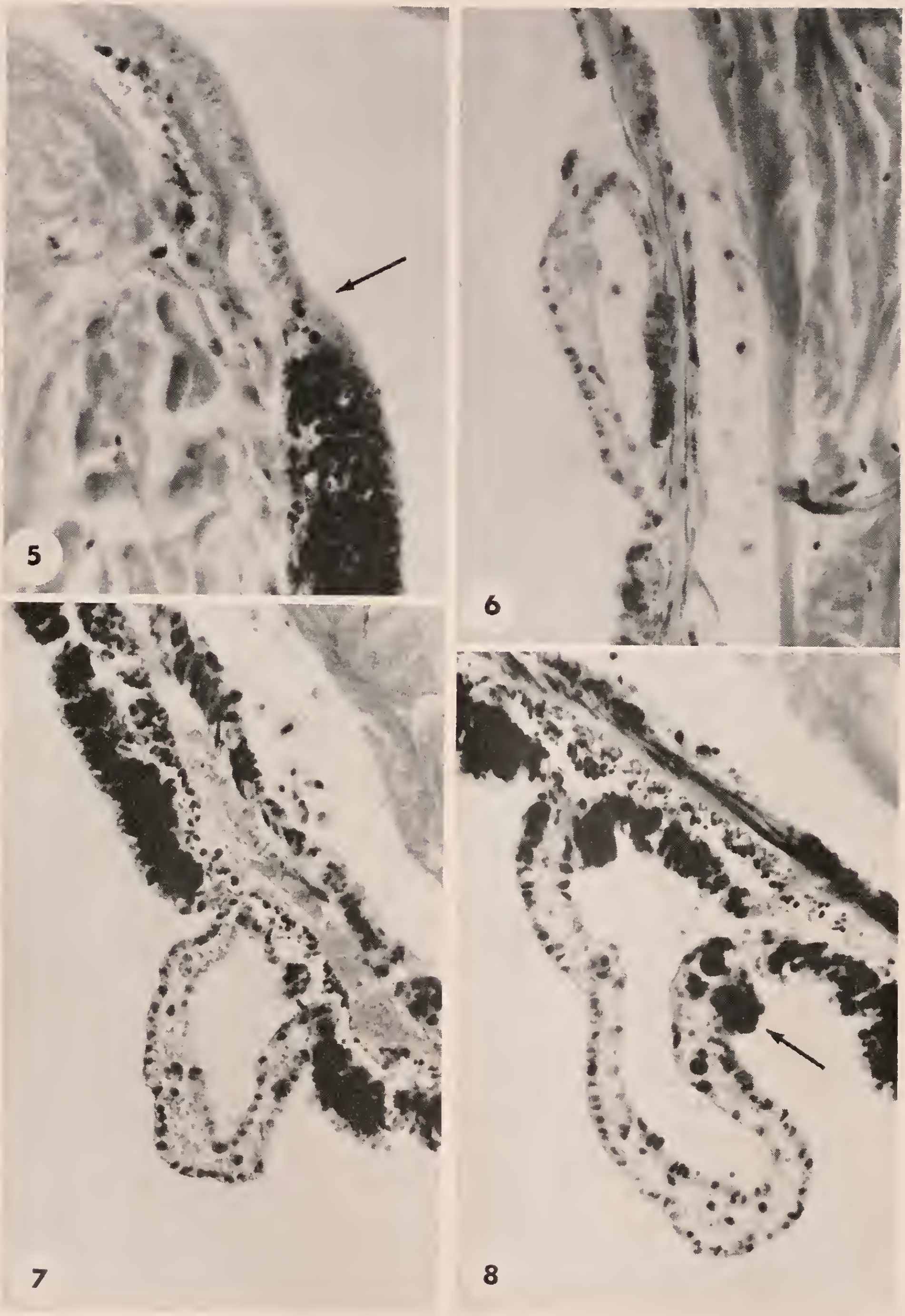
FIGURE 1. Dissection of a fresh specimen, viewed from oral aspect, showing proximal portions of intact digestive organs in one ray. Tp = Tiedemann's pouch; arrows indicate approximate level at which the organs were severed in operative removal. Approximately 10 ×.

FIGURE 2. Cross-section of ray showing relationships of body wall, mesenteries, and pyloric caecum. Aboral body wall at left; L = lumen of median caecal duct; arrows indicate portions of the paired suspensory mesenteries, which are not continuous sheets but interrupted webs. Harris' hematoxylin, fast green. 230 ×.

FIGURE 3. Hypertrophied, granule-packed peritoneum in a regenerating specimen, fifth postoperative week, at a distal level in the ray; compare with normal peritoneum in Figure 2. Phosphotungstic acid (PTA) hematoxylin. 460 ×.

FIGURE 4. Taller hypertrophied peritoneum at a more proximal level in a specimen after four weeks' regeneration. Periodic-acid-Schiff, fast green, Weigert's hematoxylin. Note that the cytoplasmic granules are PAS-negative, while small areas in the free ends of the cells react strongly with the Schiff reagent. 460 ×.



Figures 5–8.

granules normally present in zymogen cells of the caecal epithelium. It is to be noted that hypertrophy of the peritoneum is confined to the general coelomic lining and never involves the mesothelial cells which form the lining of the dermal branchiae; there is an abrupt transition where the general parietal peritoneum joins the mesothelium at the base of each papula (Fig. 5).

The other notable change at the close of the second postoperative week involves the fusion of the remnants of the paired caecal mesenteries. The members of each pair have become attached to each other by their free edges to form a mesenteric tunnel, which distally is small and flattened (Fig. 6) but proximally becomes progressively taller as it approaches the stump of the caecum (Figs. 7, 8). Unlike the fenestrated, weblike mesenteries noted in the normal animal, the regenerating mesenteric tunnels clearly consist of continuous mesothelial sheets enclosing between them a loose mesenchymal layer. Proximally, the mesenteric tunnels join the suspensory mesenteries of the transected pyloric ducts remaining from the operation. Throughout their length, the mesothelia of the tunnels are continuous with the parietal peritoneum. The fact that, as seen in Figure 8, granule-filled peritoneal cells are found partway up the side of a tunnel suggests that the mesenteries incorporate cells from the general peritoneum at their bases. The mesenchyme enclosed between the mesenteric sheets communicates basally with the subperitoneal muscular and collagenous layers of the body wall. It consists of a loose, fibrous meshwork containing large cells, many of which contain deeply-staining cytoplasmic inclusions.

Proximally, the stumps of the transected pyloric ducts have, in effect, simply rounded up and healed over. In one case (Fig. 9) the zymogenic epithelium characteristic of the roof of the pyloric duct has formed a tube which extends a short distance beyond the beginning of the mesenteric tunnel before ending blindly. In none of these proximal remnants are there indications that new growth has occurred in the two weeks since removal of the caeca.

Grossly examined, the single specimen opened three weeks after caecal extirpation shows that the proximal stumps remaining after the operation have produced a short outgrowth extending in one of the mesenteric tunnels. Sections of these regions reveal that the remnants are still histologically recognizable as parts of Tiedemann's pouches and the radial reservoirs of the pyloric stomach, twisted and distorted (Fig. 10). Aborally, a tubular structure separates from the proximal stump (Fig. 11) and proceeds distally, pushing its way through the mesenchyme at the summit of a mesenteric tunnel, gradually tapering, becoming a solid rod of cells, and finally terminating after a course of a few millimeters. The second mesenteric tunnel separates from the basal remnant and extends into the ray without an epithelial tube or rod at its summit. Near its proximal end, the epithelial tube that is present shows histological characteristics reminiscent

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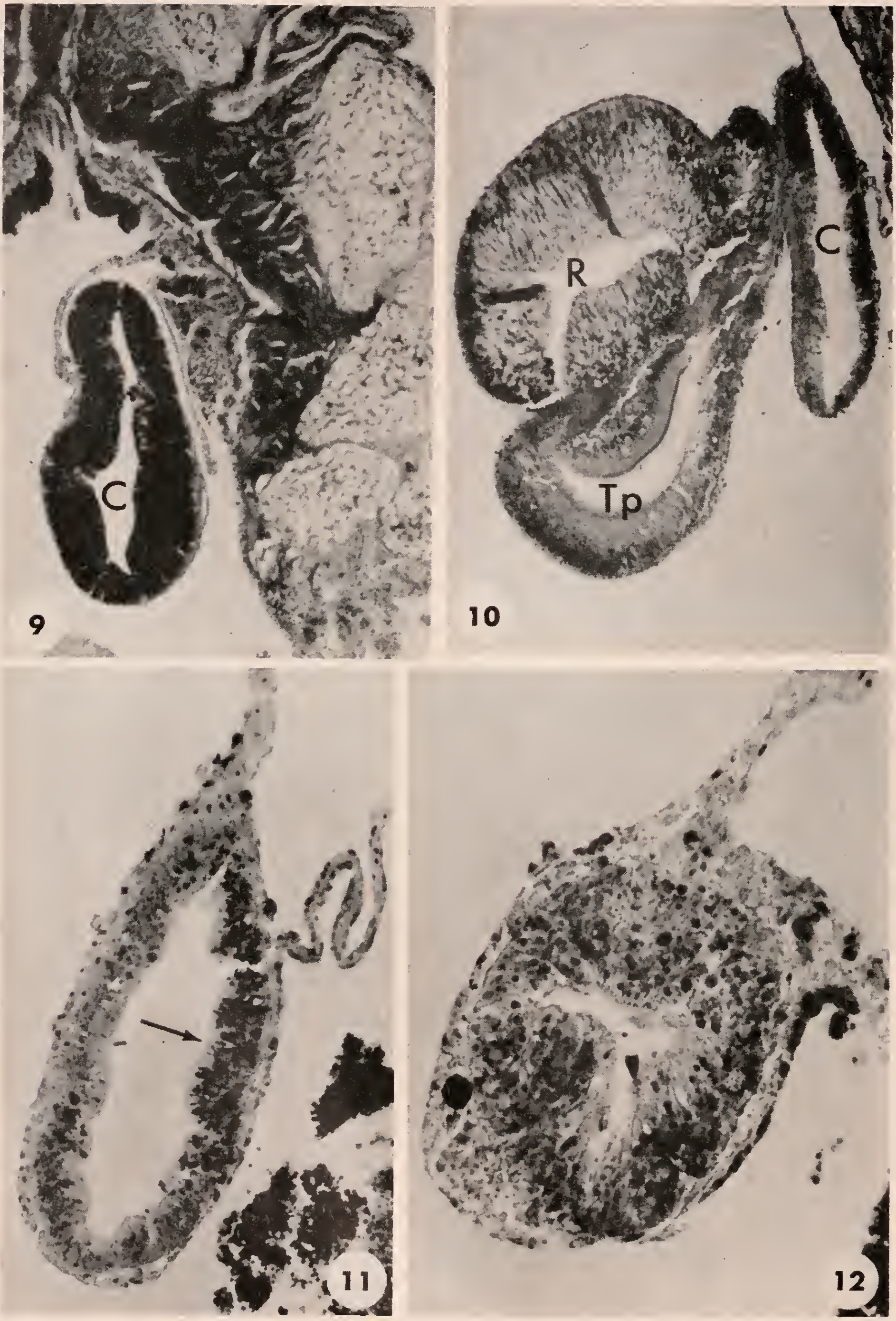
FIGURE 5. Section showing sharp transition (arrow) between hypertrophied general peritoneum and the mesothelium lining a dermal branchia, in a specimen after 3 weeks' regeneration. PTA hematoxylin. 460×.

FIGURES 6, 7, 8. Progressively more proximal cross-sections of the mesenteric tunnel formed in a two-week postoperative specimen by fusion of the free edges of paired suspensory mesenteries. Note that the mesenteries now constitute continuous mesothelial sheets separated by loose mesenchyme. The flat distal tunnel becomes progressively higher at proximal levels. Arrow (Fig. 8) indicates a granular peritoneal cell in mesothelium of the tunnel. All PTA hematoxylin. 460 ×.


C
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Figures 9–12.

of the median duct of the pyloric caecum, especially in the concentration of cells resembling zymogen cells in its roof (Fig. 11). It is to be noted, however, that these epithelial cells and the crowded spherules they contain are disorganized and preserve none of the very regular orientation normally found in the roof epithelium of the median duct. In the regenerate, these cells appear to be resorbing their secretion, rather than producing it as in the normal animal. Following the sections distally, one finds that the tube becomes progressively smaller and more nearly cylindrical, and the lumen steadily decreases in size. In these regions the nuclei of the epithelial cells are crowded basally, leaving the apical ends of the cells relatively clear (Fig. 12) ; there are some indications here of the development of a brush border and other apical specializations, as in the normal caecal epithelium. The terminal, rodlike portion of the outgrowth, beyond the point at which the lumen disappears, is composed of packed, spherical, largely undifferentiated cells. A few cells at this level contain large mucous vacuoles, but there are no indications of other types of secretory activity. The sections show clearly (Figs. 12, 13) that in three weeks the regenerative process has produced only an epithelial outgrowth advancing in the mesenchyme between the mesothelial sheets forming the mesenteric tunnel. As shown in Figure 14, this mesenchyme contains numerous cells that appear to be undifferentiated amoebocytes, some with cytoplasmic inclusions of various kinds. While the line of separation between the epithelial core of the regenerate and the surrounding mesenchyme is clearly distinguishable, no "basement membrane" (= collagenous layer; see Ferguson, 1960) has been laid down. In fact, none of the normal subepithelial components of the caecum, such as the nerve plexus layer, muscular layers, etc., are yet represented in the three-week specimen. Beyond the distal end of the rodlike outgrowth, the mesenteric tunnel continues a long distance into the ray; it here appears similar in all respects to the tunnels noted as the only regenerative products in the two-week specimen (Fig. 15).

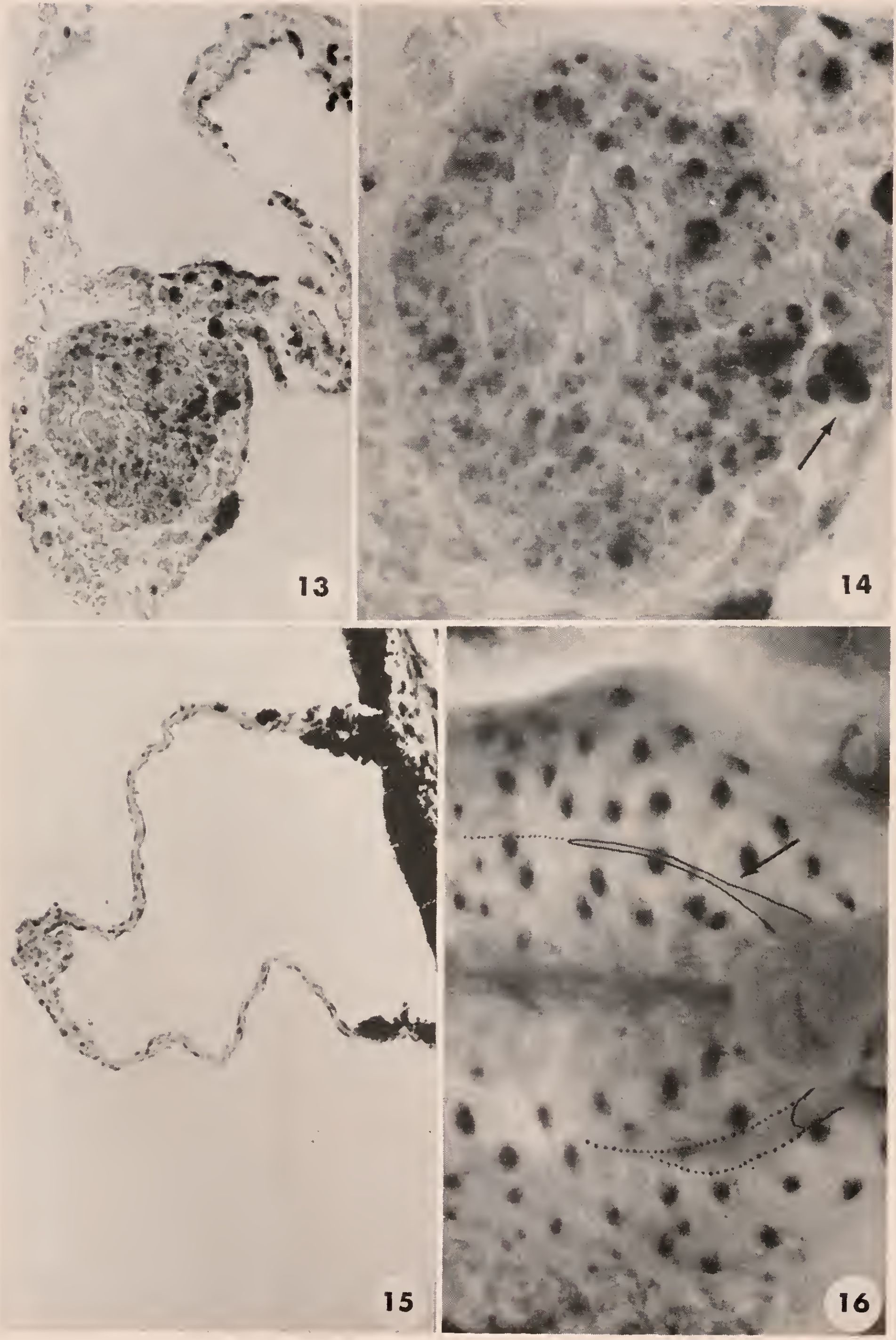
An overall view of conditions in the operated ray of a four-week specimen is shown in Figure 16. Basally, the remnants of the paired Tiedemann's pouches and radial reservoirs have shrunken and adhered to one another, forming a single twisted fusion mass. From the mesenteric supports of this basal structure two mesenteric tunnels lead outward into the ray. One of these (the lower, in the photograph) is small, flat, rather poorly developed, and appears empty except as it is associated with a short, blunt projection from the corner of the fusion mass.

Figure 9. Cross-section of healed stump of median caecal duct (C), two-week specimen. Aboral body wall at upper right. Note the typical high concentration of zymogen cells in the epithelium of the duct. PTA hematoxylin. Approximately 75 ×.

Figure 10. Cross-section of regenerating specimen at proximal level, three weeks postoperative. At R and Tp, recognizable remnants of radial reservoir and Tiedemann's pouch are indicated. Above these, C denotes the tubular caecal regenerate at the level where it begins to separate from the proximal remnants of the operated organs. This tube arises aborally, from the stump of the median caecal duct. PTA hematoxylin. Approximately 75 ×.

Figure 11. Cross-section of the same three-week tubular regenerate at a more distal level. Note concentration of disorganized zymogen-cell remnants in roof of tubule (arrow) ; mesenteric attachments at upper right. PTA hematoxylin. 230×.

Figure 12. Cross-section of the same tubular regenerate (three weeks postoperative) still more distally. The lumen is poorly developed at this level, but the epithelial cells show conspicuous signs of differentiation. Note the sharp line of demarcation between the bases of the epithelial cells and the surrounding mesenchyme. PTA hematoxylin. 460 ×.



FIGURES 13–16.

The other, however, is occupied by a tapering extension from its corner of the mass, and this accompanies it for a considerable distance into the ray. The relationships of these parts are best understood by studying serial cross-sections of the material. The histological characteristics of the basal fusion mass remain recognizably those of the radial reservoirs and Tiedemann's pouches; the less well developed of the two mesenteric tunnels is observed to lead off from its corner of this structure and almost immediately to become flattened against the aboral body wall. Throughout its subsequent course it remains undifferentiated, resembling very closely the two-week distal tunnel shown in Figure 6.

The outgrowth accompanying the second tunnel is a tubular structure arising from the most aboral part of the basal remnant. For a short distance on its oral side its layers are continuous with those of the Tiedemann's pouch remnant lying below it, but judging from its point of origin as well as from its histological characteristics, this tube clearly represents an outgrowth from the transected median duct of the corresponding pyloric caecum. As Figure 17 shows, the origin of this tube is similar to that of the three-week regenerate; at four weeks, however, the epithelium contains no significant concentrations of the deeply-staining secretory material characteristic of the normal median-duct epithelium and noted aborally in the three-week specimen (compare Figs. 11 and 17). In other respects the epithelium at this proximal level has undergone considerable differentiation (Fig. 18). Mucous gland cells are well represented, and the majority of the ordinary epithelial cells have begun to assume more of the normal columnar aspect, with nuclei crowded towards their bases and the distal ends showing signs of incipient flagellum and brush-border differentiation. It is noteworthy that the tubular regenerate in this proximal, most advanced level is tall and narrow; this suggests that the deepening of the radial organ, bringing about separation of the oral, outflowing flagellary currents from the aboral, inflowing ones, has already begun in the fourth postoperative week. This deepening has, to be sure, advanced only a limited distance in the regenerate; beyond the approximate point indicated by the arrow in Figure 16 the tubule tapers to a cylindrical cross-section, and progressively more of the regenerate comes to be occupied by epithelial cells as the lumen gradually disappears. At this level (Fig. 19) the four-week regenerate is practically identical in composition and appearance with the more proximal levels of the three-week regenerate (compare Fig. 12); the cells are relatively undifferentiated, crowded basally but elongating distally, and numerous mucous goblets are present. Beyond the point at which the lumen disappears there is a

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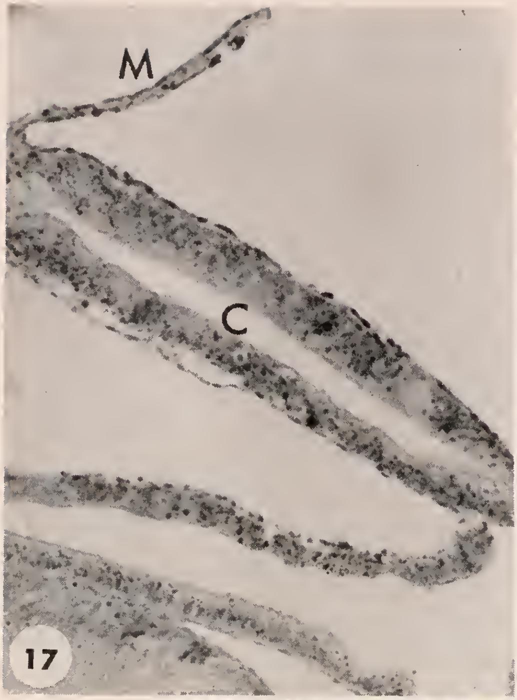
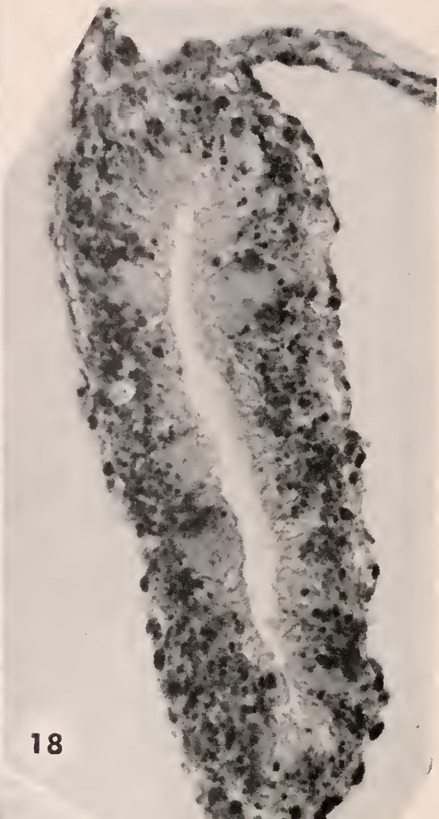
FIGURE 13. Cross-section of three-week regenerate at still more distal level, where the advancing rod has not yet developed a lumen. The distinction between epithelial outgrowth and the surrounding mesenchyme is clear. PTA hematoxylin. 460 ×.

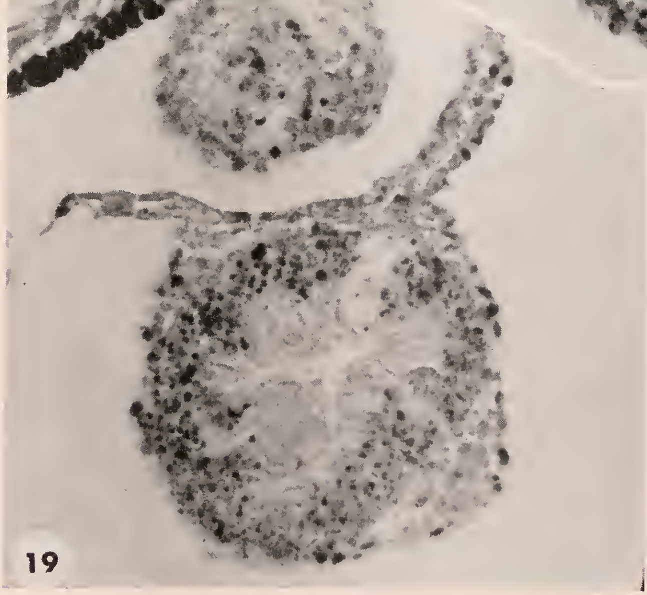
FIGURE 14. Same section as in Figure 13, somewhat enlarged. The clear intracellular areas in the mass of epithelial cells are mucous secretions. Arrow indicates a clump of amoebocytes with coarse granular inclusions. PTA hematoxylin. 1100 ×.

FIGURE 15. Cross-section of mesenteric tunnel in three-week regenerate beyond the level reached by the advancing epithelial rod. PTA hematoxylin. 230 ×.

FIGURE 16. Dissection of fresh specimen showing conditions at the close of the fourth postoperative week; center of disk is to the right. At the base of the ray, note the twisted mass representing fused remnants of Tiedemann's pouches. The extent of the two regenerating caeca is indicated by inked outlines; the mesenteric tunnels are dotted. Arrow indicates the point at which the tall, narrow regenerate tapers to a simple cylindrical tube. Approximately 15 ×.







FIGURES 17–19.

short, solid, rodlike region of the outgrowth similar in all respects to the three-week level shown in Figure 13; past its termination, a well-developed mesenteric tunnel can be traced a considerable distance out into the ray.

One further observation on the four-week specimen is of significance: the regenerate does not contain, at any level, recognizable representatives of the normal subepithelial components of the caecum. The space between the base of the lining epithelium and the covering visceral peritoneum is still occupied only by loose mesenchyme containing amoebocytes.

At six weeks (Fig. 20) the regenerate is larger and longer than at four. In this specimen, one of the two regenerating tubes was unfortunately damaged in preparation, and the tissue was not sectioned. It is apparent from general examination, however, that although the damaged tube was not as long as the intact one it was relatively well developed. Thus, unlike the situation in specimens sacrificed earlier, both of the caeca were being replaced in this six-week animal, one perhaps more slowly than the other. In other respects appearances indicate that regenerative progress at six weeks is about what might have been predicted from study of the earlier stages.

Figure 21 shows the regenerating caeca in the most advanced of the specimens operated in this series, sacrificed eight weeks after caecal extirpation. The paired stumps of Tiedemann's ducts and the radial reservoirs have not healed together as in the four-week specimen; rather, each has retained its integrity and has produced an extension into the ray. Again, one of these is somewhat in advance of the other. The regenerating structures take their origins from the aboral parts of the basal remnants, although their layers are proximally continuous with those of the Tiedemann's ducts as well. Histologically, this eight-week specimen shows several interesting and significant features; attention will be focused on the better-developed of the two regenerates. Near its origin, this structure is a tall and narrow tube, with a lumen bounded by markedly normal-looking columnar epithelium (Fig. 22). Proximally, the roof of the tube shows a considerable concentration of cells containing strings of zymogen granules, scattered among the typical attenuated epithelial cells (Fig. 23). Close study of this region shows that the epithelium is flagellated, as in the normal caecum. The floor of the tubular regenerate is occupied by crowded cells with long flagella, forming a gutter-like structure. In these cells, and in those lining the sides of the tube, concentrations of moderately fine PAS-positive droplets are localized. Such droplets stain similarly to the contents of mucous gland cells, which are also numerous in this region, and may represent precursors of mucous secretions. The differential concentration of zymogen cells aborally, and of mucous-related and current-producing cells orally, suggests re-establishment of the normal regional

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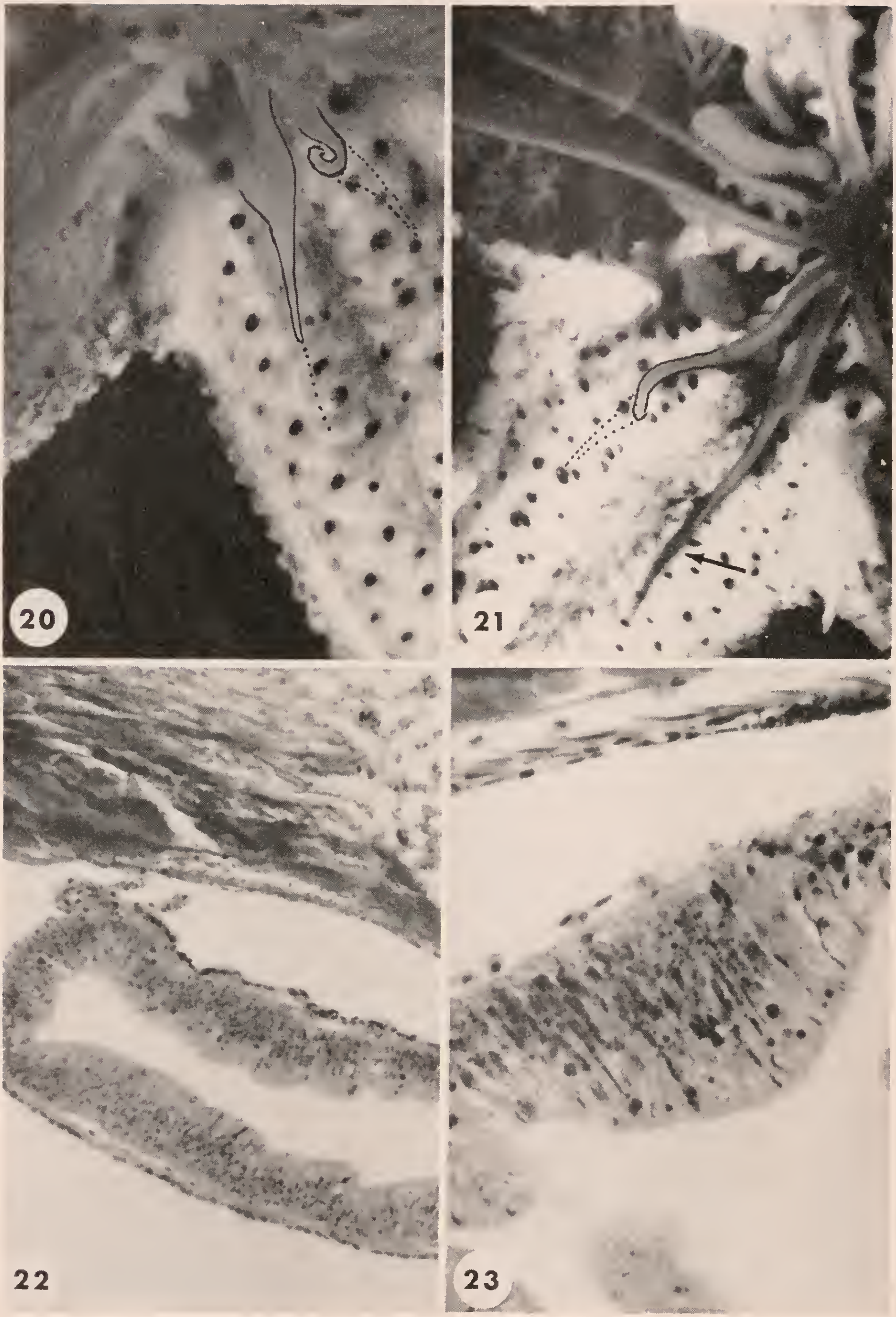
FIGURE 17. Section at proximal level in four-week regenerate. M = one of the mesenteric sheets; C = caecal regenerate. At lower left a portion of the basal remnant of Tiedemann's pouch. PTA hematoxylin. 230 ×.

FIGURE 18. Section at a more distal level in four-week regenerating caecum; mesenteric attachments above. Note the considerable cellular differentiation in the epithelial lining, with occasional mucous goblets (clear areas) and basal concentrations of nuclei. No subepithelial components (muscle, connective tissue, etc.) have yet appeared. PTA hematoxylin. 460 ×.

FIGURE 19. Four-week regenerate sectioned at a level just beyond the extent of the lumen; compare with section of three-week regenerate in Figure 12. Mucous glands well represented. A fragment of debris is trapped in the mesenteric tunnel above the regenerate. PTA hematoxylin. 460 ×.



Figures 20–23.

specializations of the caecum and its pouch. In these more advanced proximal regions a definite subepithelial collagen layer is now recognizable, and the characteristic network of muscle fibers is also in evidence. These layers are not fully developed, and there are no clear indications of a nerve plexus layer, but conditions here are much advanced when compared with those in the four-week regenerate. The visceral peritoneum is thin and flattened, and its nuclei are again normally bean-shaped. Notably, the hypertrophy of the parietal peritoneum has subsided, and there are only scattered areas in the vicinity of the mesenteries, as in Figure 23, where cells of this layer are still charged with deeply-staining spherules.

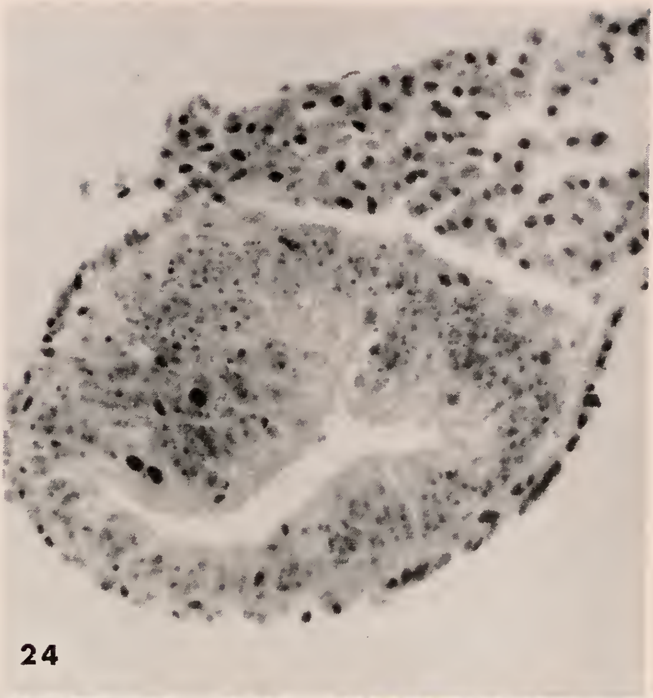
At progressively more distal levels the characteristics of the eight-week regenerate show gradual changes toward those of less advanced stages. Approximately halfway between base and tip, for example, the shape of the epithelial tube has become more nearly cylindrical, and its lumen is relatively restricted. The cells forming the tube are on the average tall and well differentiated, but no zymogen cells are in evidence. There appears, however, as in Figure 24, a noticeable distinction between the thick-walled roof and the thin-walled floor of the tube at this level. Subepithelial components do not extend to this point in the regenerate. Even farther distally, the lumen disappears altogether, and the regenerate is represented only by a solid, rodlike outgrowth in the mesenchyme of a mesenteric tunnel. Beyond the end of this solid rod the tunnel continues for some distance, very similar in essential features to tunnels observed earlier. One aspect of this specimen is noticeably different: the distal, tapering portion of the longer regenerate appears to be surrounded by a diffuse, granular mass which in the living specimen was colored a vivid orange (Fig. 21). In sections, this mass is revealed as consisting of an extensive accumulation of coelomocytes, apparently trapped inside the mesenteric tunnel (Figs. 24, 25). The relationship between this coelomocyte clot and the tissues of the regenerate and its supporting tunnel is so close as to suggest that coelomocytes may have contributed to the cells incorporated in the regenerating tissue. Far distally, beyond the region of coelomocyte accumulation, the mesenteric tunnel extends as a simple, low structure identical in appearance and composition to the tunnels observed, for example, in the two-week specimen (compare Figs. 6, 26). At this distal level it will be noted that the parietal peritoneum retains considerable patches of hypertrophic cells packed with granules similar to those found more proximally in younger stages.

FIGURE 20. Fresh dissection of specimen at close of sixth week of regeneration. The tapering, tubular caecal regenerates are outlined in ink, with mesenteric tunnels dotted. The curled regenerate was damaged in the dissection. Note persistence of scar in the area of the healed incision (between the two regenerates). Approximatly 15 ×.

FIGURE 21. Fresh dissection of specimen after eight weeks' regeneration; one of the regenerates and its mesenteric attachment emphasized by inked outline. The levels at which the organs were originally severed are clearly shown as the origins of the somewhat smaller regenerating tubes. Arrow indicates concentration of coelomocytes described in the text. Approximately 10 ×.

FIGURE 22. Cross-section at proximal level through eight-week regenerate; note normal appearance of the epithelium. The tubule is tall and narrow, with its roof at left; it has swung to the right in processing. PTA hematoxylin. 230 ×.

FIGURE 23. Details of epithelium in the roof of eight-week regenerate, showing accumulation of apparently functional zymogen cells. The epithelium is flagellated, and subepithelial collagenous and muscular layers are present at this level. PTA hematoxylin. 460 ×.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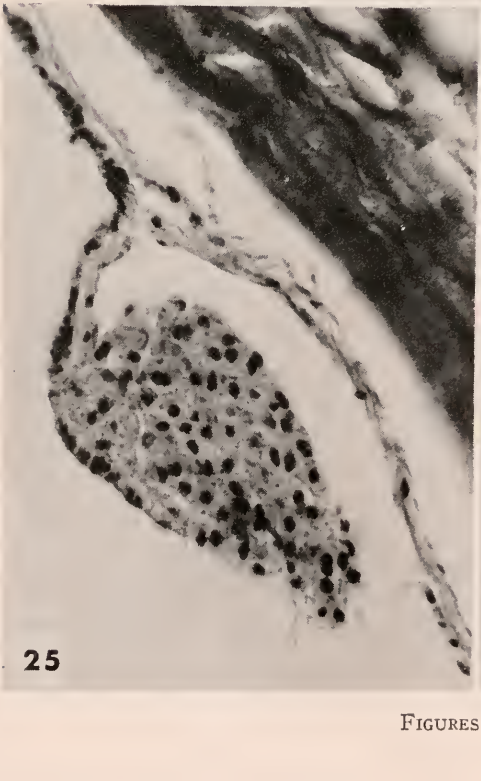
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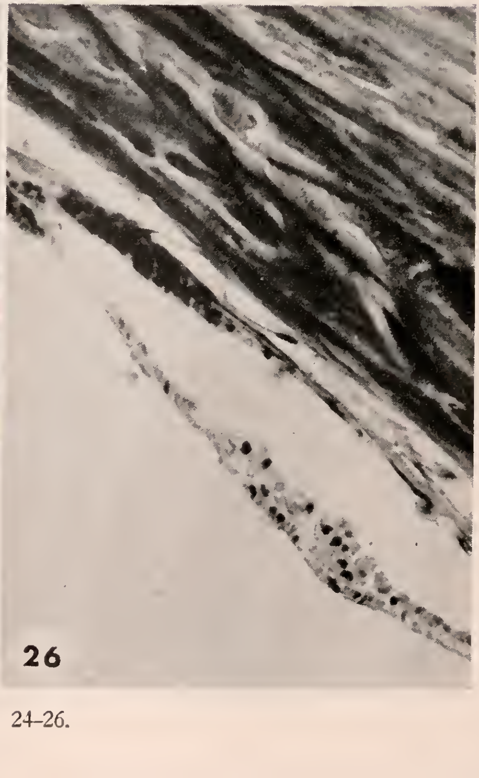
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Figures 24–26.

DISCUSSION

The experiments described here establish the fact that within the otherwise undamaged ray of a sea-star, after healing of the operative incision, excised pyloric caeca are replaced by regenerative processes following a consistent pattern. As pointed out by Hyman (1955) in the passage quoted earlier, when an entire ray is being regenerated the specializations characteristic of the distal extremity are produced first. Just proximal to the newly formed tip lies a localized zone of growth which moves outward, pushing the tip ahead of it and generating replacement tissues behind it in the elongating ray. Thus, as clearly demonstrated in the work of Schapiro (1914) and Zirpolo (1921), the oldest, first-differentiated region of a regenerating ray is the distal tip; immediately adjacent to this lies the least-differentiated region, constituting the zone of growth, and between this zone and the base of the regenerating ray there is a gradient of increasing age and differentiation in the tissues. These facts have been demonstrated for the parietal portions of the ray; in contrast, the present studies show that in the regenerating pyloric caecum of *Henricia* there is no differentiated tip region proceeding distally in advance of a growth zone. The tip of the regenerating caecum, at the extremity of the solid rod of cells pushing outward in the summit of the mesenteric tunnel, is composed of the least-differentiated and presumably youngest cells, and the gradient of increasing differentiation begins immediately behind this tip. The advancing tip of the rod thus appears to constitute, or at least to include, any zone of growth that may be present in the caecal regenerate. To this extent, then, the growth processes involved in replacing excised visceral components of the ray differ markedly from those producing parietal or body-wall portions. It seems reasonable to infer that these differences prevail also in the normal growth of the sea-star and in situations involving regeneration of entire rays following autotomy.

The remnants of the mesenteries are evidently of great significance in the replacement of excised pyloric caeca. In no case have I observed regeneration of a caecum in the absence of the characteristic preliminary changes involving the suspensory mesenteries. The initial reactions of the mesenteric remnants are particularly conspicuous in *Henricia*, where the mesenteries are normally discontinuous and web-like; here, one of the earliest internal events leading to caecal regeneration involves the formation, from these tattered strands, of the continuous, double-mesothelial sheets that join in pairs to constitute the mesenteric tunnels. The proximal portions of these tunnels, with the mesenchymal elements enclosed between their mesothelial layers, are in place and in readiness by the time the initial outgrowths from the proximal stumps of the caeca begin. The rod of cells advancing distally into the ray from the stump derives guidance and support from the mesenteric tunnel; further, it appears highly likely that at least some of the histological components of the regenerating caecum may originate in, or pass through, the mesenchyme filling the space between the mesothelial layers of the tunnel.

FIGURES 24, 25, 26. Progressively more distal levels of eight-week regenerate. Above the tubular regenerate in Figure 24 the mass of coelomocytes trapped between the mesenteries is in contact with the subepithlial mesenchyme and may be contributing cells to the regenerate. Conditions at the far distal levels in the eight-week specimen are comparable to those found more proximally in younger regenerates. All PTA hematoxylin. 460 ×.

The cause and possible significance of the marked hypertrophy of the peritoneum in regions adjacent to the mesenteric tunnels, and the nature and source of the cytoplasmic spherules with which the hypertrophied cells are packed, remain obscure. The development of the hypertrophy follows soon after operative removal of the caeca, but it may be related to the processes of healing and repair of the incision in the body wall rather than to regenerative changes leading to replacement of the caeca. The simple experiment which might shed light on this point has not been performed. In either case, two alternative explanations suggest themselves to account for the accumulation of cytoplasmic spherules in the peritoneal cells. Perhaps the spherules represent condensed remnants of digested tissue fragments, debris, necrotic material, and the like taken into the peritoneal cells by phagocytosis and held here either for eventual elimination or for ultimate utilization in the processes of repair. Alternatively, they may represent nutritional reserves mobilized and concentrated in the peritoneal cells nearest the sites of repair and regeneration. No attempts at precise characterization have been made, but the spherules are evidently neither fatty nor of predominantly polysaccharide nature; their general staining behavior resembles that of some proteins. Spherule-packed peritoneal cells are frequently incorporated into the mesothelial sheets forming the mesenteric tunnels. At early stages this is particularly noticeable in proximal areas near the severed caecal stumps, where degenerative changes in the caecal epithelium seem to be occurring. Later, peritoneal hypertrophy has usually subsided in the proximal regions where regeneration of the caeca is well advanced, but it persists in the more distal areas in association with earlier regenerative phases.

The close relationship which exists in *Henricia* between regenerating caeca and the remnants of the mesenteries suggests comparison with a parallel situation previously described by several investigators studying visceral regeneration in various sea-cucumbers. In all the holothurians studied, replacement of the gut always involves an initial thickening of the free edge of the mesentery. In *Stichopus*, studied by Bertolini (1930) and by Dawbin (1949), the thickening develops at a practically uniform rate along the length of the mesentery and results, according to Dawbin, from an increase in the number of mesenchyme cells enclosed between the mesothelial sheets which have fused at the mesentery edge, where the mesenchyme gradually forms a solid, cordlike swelling. In *Thyone*, Kille (1935, p. 93) states, without giving additional histological details, that sections of the thickened edge of the mesentery show it to be ". . . a solid rod of connective tissue covered by a much thickened mesothelium." Whether this thickened mesothelium is like that encountered in *Henricia* cannot be determined from this description. In various species of *Holothuria*, Bertolini (1932) and Kille (1936) have described early thickening of the mesentery edge involving hyperplasia of the mesenchyme. In all the sea-cucumbers studied, it is within this rodlike swelling at the mesentery edge that the lumen of the new gut forms, but there is a surprising divergence in the processes leading to its formation in the various genera. In *Stichopus*, Bertolini and Dawbin agree that the lumen develops by extension and fusion of clefts in the mesenchymal thickening, and that its lining forms by later differentiation of mesenchymal cells. In *Thyone*, and even more clearly in *Holothuria*, the lumen of the new gut does not develop directly in the mesenchymal thickening at the mesentery edge; this thickened edge is secondarily invaded by tubular ingrowths from both ends. In *Thyone*, which eviscerates orally ("anteriorly"), only the posterior tongue of invading cells is continuous with a remnant of the original gut; the anterior ingrowth stems from a portion of the newly regenerated lantern. In *Holothuria*, which eviscerates aborally, both of the ingrowing tubules originate from remnants of the old digestive tract. The lumens of these tubules become continuous where the ingrowths meet and fuse, and the origin of the lining epithelium is clearly different from that found in *Stichopus*.

This contrast in regenerative processes has been recognized by several students of holothurian gut regeneration and has been most recently discussed by Dawbin (1949). What is of particular interest in the present connection is the general similarity between the process of gut regeneration in *Holothuria* and that now described for the replacement of pyloric caeca in *Henricia*. The differences in detail that do exist can be related to differences in the structure of the mesenteries in the two forms (single in *Holothuria*, paired in *Henricia*), and perhaps more particularly to the fact that in the sea-star we are dealing only with radial blind pouches of the gut rather than, as in the holothurian, with the entire axial portion of the digestive tract. But the replacement of the pyloric caecum in *Henricia* follows the same sequence of events as that displayed in the replacement of the gut in *Holothuria:* fusion of mesothelia at the free edge of the mesentery, followed by thickening of the mesenchyme enclosed here; invasion of the mesenchymal rod thus formed by tongues of cells advancing from whatever stumps of the original structure remain, a lumen then developing inside the ingrowth; and, finally, differentiation of a normal lining epithelium and of the subepithelial components of the organ.

The rod or tongue of tissue growing outward from the stump of the pyloric duct in *Henricia* advances in such a way as to suggest that an active center of cell proliferation must be present in the regenerating organ. Further, the gradient of differentiation in the newly forming caecum, running from tip to base, indicates that the source of the cells must lie at or near the tip itself, growing distally and leaving new cells behind it to undergo differentiation. Surprisingly, however, no zones of mitotic activity have been found in any of the regenerating caeca studied, although such areas have been carefully sought. Perhaps cell divisions in this tissue occur in cycles, and my observations have by chance missed what is in fact occurring. Alternatively, there is the possibility that the regenerating tissue grows not through the proliferation of its own cells but by the incorporation of amoebocytes recruited from the myriads wandering in the mesenchyme throughout the body. The presence of an abundance of large amoebocytes in the mesenchyme of the mesenteric tunnels has been noted, and it may be that one of the important functions of the mesenteries is to form a bridge for the passage of such cells from the body wall into the region of the advancing tubular regenerate. In *Stichopus*, Dawbin (1949) describes numerous large amoebocytes passing by way of the mesentery into the regenerating gut, and accumulations of these cells form the thickening at the mesentery edge. Only in later stages, after the formation of the lumen, does mitotic activity begin to increase the numbers of cells destined to form the lining epithelium.

This account by Dawbin, it should be noted, is the only treatment of regeneration in echinoderms in which I have found specific reference to cellular proliferation in the regenerating tissue, and the situation in *Stichopus* is so different from that in other holothurians, and in asteroids, that it may present no valid basis for comparison. It would be interesting to know the source of cells making up the tongues of tissue invading the mesenteries of *Holothuria*, but Bertolini's description (1932) unfortunately fails to make clear whether mitotic activity is involved. Turning from visceral regeneration to the more familiar and presumably better understood matter of parietal regeneration in asteroids, the account by Nusbaum and Oxner (1915) of regeneration in *Echinaster* describes histological details of wound healing and the reconstitution of muscular and skeletal elements. Outlining such events as dedifferentiation of specialized structures, phagocytosis of debris, and the like, the authors conclude that the principal processes involved in regeneration depend upon the mobilization, incorporation, and differentiation of amoebocytes. They apparently assign no role to cellular proliferation, and if they observed it at all this fact is not mentioned.

Obviously, problems of regeneration in asteroids, both visceral and parietal, require further study with attention to histological details and with more refined methods; equally obviously, the available evidence is too meager to form the basis of firm conclusions. But it seems at least admissible, and in view of the information available for other regenerating echinoderms not unreasonable, to suggest that in the regenerating pyloric caecum of *Henricia* there is no zone of growth in the sense of a localized distal region of mitotic activity. Until further studies now in progress to elucidate this point can be completed, it is provisionally concluded that the advance of the undifferentiated tip of the regenerate may occur by progressive accumulation and addition of amoebocytes moving through the mesenchymal layer. Further conjecture at the moment seems unwarranted.

It is of interest to note that Tiedemann's pouch does not form in the regenerating caecum by outgrowth on a broad front from the stump of what has been termed Tiedemann's duct (Anderson, 1960). Rather, in its early stages the caecal regenerate is simply tubular and even at eight weeks forms a simple cylinder in its younger, more distal portions. As has been noted, however, even at four weeks the proximal portion has become much deeper in cross-section, and by the eighth week there are histological indications in the floor of the basal region suggesting that differentiation of the current-producing areas of the epithelium has begun. The localization and concentration of apparently active zymogen cells in the aboral epithelium of the regenerate is most conspicuous, and the contrast between roof and floor gives a clear forecast of the functional differences to be found in the finished organ. It is clear, however, that Tiedemann's pouch arises by progressive differentiation of the floor of the tubular regenerate. The cytological details of the transformation of undifferentiated epithelial cells into such highly specialized elements of the normal epithelium as zymogen cells, mucous gland cells, and the like remain to be described.

SUMMARY

1. Regeneration of pyloric caeca has been studied grossly and histologically in a series of specimens sacrificed one, two, three, four, six, and eight weeks following operative removal of the caeca by way of a median longitudinal incision through the aboral body wall of one ray.

2. After healing of the incision, which is fairly complete at the close of the second week, the cut stumps of the caeca are found to have healed over. The original web-like suspensory mesenteries have become continuous mesothelial sheets and have fused in pairs at their free edges to form mesenteric tunnels leading outward into the ray. The parietal peritoneum has become conspicuously hypertrophic, its normally flattened cells now tall and filled with deeply-staining cytoplasmic spherules.

3. During subsequent weeks one or both of the mesenteric tunnels may be invaded by an outgrowth from the central stump of the caeca, the outgrowing tongue of cells pushing through the mesenchymal thickening at the summit of the tunnel enclosed between the mesothelial layers. During early weeks one outgrowth usually lags behind the other; in later stages the two are more nearly equal in length and development. The tip of the outgrowth consists of a rod of large, undifferentiated cells sharply marked off from the surrounding mesenchyme. A short distance behind the tip a lumen develops simply as a cleft in the packed cells of the cylindrical regenerate. Nearer the base, the cells are seen to have undergone rearrangement to form a more regular lining, composed of taller cells showing the beginnings of a brush border and flagella. At all stages the outgrowth presents a regular gradient of differentiation between tip and base, the cells forming the tip being undifferentiated and young while those nearer the base show a gradual increase in degree of differentiation. At later stages this culminates, basally at least, in the formation of an essentially normal epithelium, provided with mucous gland cells and zymogen cells. Subepithelial components, such as the characteristic muscular and connective-tissue layers, are slower to form but are in evidence at the base of the regenerate by the eighth week. The normal marked distinction between oral and aboral parts of the caecum begin to be suggested also by the eighth week; it is apparent that Tiedemann's pouch will form not by outgrowth along a broad front from its remnant stump but by specializations in the floor of the tubular caecal regenerate.

4. The process of regeneration in the pyloric caecum differs in at least one important respect from that involved in replacement of the parietal parts of a regenerating ray. In the regeneration of the entire ray, the highly differentiated distal tip with its sensory specializations forms first and precedes the more proximal, less differentiated areas as the ray elongates. By contrast, in the caecal outgrowth there is clearly no precociously differentiated tip region; the tip is obviously the youngest, least-differentiated portion. The sequence of events in regeneration of the pyloric caecum resembles to a degree that which has been described for replacement of the digestive tract following evisceration in *Holothuria*.

5. A particularly puzzling aspect of the regenerative process involves the fact that no zone of mitotic activity has been found in the caecal outgrowth, although such a zone might be expected to lie near the advancing tip of the regenerate. This raises the question as to the source of cells in the newly forming caecum, and it is suggested that the cells may come not from proliferation but from recruitment and incorporation of amoebocytes gathering in the mesenchymal layer through which the regenerate grows. A more general question is raised concerning the role of cell proliferation in other cases of regeneration in echinoderms; only in the somewhat atypical case of gut-replacement from mesenchyme in the

sea-cucumber *Stichopus* is there explicit description of mitotic activity as a feature of regeneration.

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LACK OF DEPENDENCE OF THE FEEDING REACTION IN HYDRA ON REDUCED GLUTATHIONE

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Several years ago, in the course of regeneration experiments, Dr. Helen D. Park observed that hydras dropped into dilute solutions of reduced glutathione (GSH) would open their mouths widely and maintain this attitude for half an hour or more. After this was made known by Park, Loomis investigated the reaction in *H. littoralis,* and published the remarkable announcement that the feeding reaction in *Hydra* depended exclusively on the release of GSH in tissue juices of living prey, following puncture by the nematocysts (Loomis, 1955a). Additional reports and frequent references to the "specific GSH reaction" have since appeared in the literature (Loomis and Lenhoff, 1956; Loomis, 1959; Lenhoff and Schneiderman, 1959; Lenhoff, 1960, 1961a, 1961b; Lenhoff and Bovaird, 1959, 1961). The existence of specific GSH receptors has been postulated, and the properties of these organs discussed (Lenhoff, 1961a, 1961b; Lenhoff and Bovaird, 1959, 1961). GSH has been termed an "environmental hormone" (Loomis, 1955a; Lenhoff and Bovaird, 1959), and the use of *Hydra* has been suggested for its bioassay (Loomis, 1955a, 1955b). It has been asserted that only living animals with pseudocoelomic, coelomic or vascular fluids rich in GSH can serve as food for *Hydra* (Loomis, 1955a; Loomis and Lenhoff, 1956). Phylogenetic speculations have been based on these statements (Loomis, 1955a; Lenhoff and Schneiderman, 1959).

Partly as a result of frequent repetition, the story of the specific dependence of *Hydra's* feeding reaction on GSH has become widely disseminated and believed. It has been included in college textbooks of zoology (Guthrie and Anderson, 1957; Moment, 1958); in popular articles directed to the lay reader (Burnett, 1959); and in collections of helpful information distributed to teachers by biological supply houses (Lenhoff, 1960). Students who know little or nothing else concerning *Hydra* are familiar with it as an example of an animal with a specific behavioral response to a particular chemical, an external hormone. The account appears to have been accepted uncritically and without reluctance.

This report will present evidence to show that hydras are not restricted to living prey or to food animals of the Nemathelminthes or higher phyla; that the feeding reaction is not exclusively dependent on GSH in ten North American hydra species, including descendants of Loomis' stock of *H. littoralis;* and that the prolonged mouth opening produced by dilute solutions of GSH does not represent a normal feeding reaction.

EVIDENCE FROM THE LITERATURE AND FROM OBSERVATION

Loomis (1955a) clearly stated that his discovery of the specific GSH feeding reaction in *Hydra* could at last provide an explanation for the ability of hydras to distinguish living from non-living prey—since this auto-oxidizable tripeptide (GSH) is present in sufficient quantity in the reduced form only in living tissue, not in bits of meat or egg (Loomis, 1955a; Loomis and Lenhoff, 1956). Loomis (1955a) told dramatically that all attempts to induce hydras to ingest non-living food in his laboratory were unsuccessful (without artificially added GSH); and that the fact that hydras fed exclusively on living animals had been known for centuries. However, it appears that the references cited by Loomis in support of these statements have been misrepresented, and that the literature abounds with descriptions of the ingestion of non-living foods by hydras.

Although Trembley (1744) was cited by Loomis (1955a) as the first to describe the exclusive dependence of hydras on living prey, Trembley actually reported successfully feeding his animals on beef and mutton (Trembley, 1744; Baker, 1952). He did not find animals eating these in nature, but this cannot be taken to support the contention that they were unable to ingest such particles for lack of GSH.

Hyman (1940) was credited by Loomis (1955a) with the statement that hydras could feed on many small aquatic species but would not feed on dead specimens of these same species. This will not be found in the reference named or in any of Dr. Hyman's numerous publications on hydras. In personal interview, Dr. Hyman stated that she had never undertaken such experiments, but would have expected the opposite result.

Whitney (1907) has been represented (Loomis, 1955a) as pointing out the lack of motility of non-living particles as a possible explanation of hydras' acknowledged restriction to living prey. Nothing resembling this suggestion appears in that reference. On the contrary, Whitney described the abundant supply of green hydras he found at the outlets of the fish pens at Cold Spring Harbor, where bits of shredded beef heart fed twice daily to the fishes were "eagerly" seized by the hydras, which flourished on this rich source of food.

Beutler (1926) was correctly reported (Loomis, 1955a) to have dipped bits of fibrin and gelatine in crustacean juice to insure ready ingestion by hydras. However, Beutler conducted no tests on the ingestion of other substances without crustacean juice, and make no general statements regarding feeding in hydras (Beutler, 1924, 1926).

Other references to the feeding of hydras on meat and egg are not difficult to find. Greenwood (1888) found that his hydras would "greedily" take up fragments of muscular fiber or spleen. Wagner (1905) successfully fed hydras raw meat. Haase-Eichler (1931) conveniently fed small bits of beef, keeping the water in motion so that pieces would come in contact with the tentacles. Pickens (1933) referred to Kepner's successful culture of hydras on beef, and to the responsiveness of the hydras to this food. Hefferan (1901) routinely fed large pieces of hard boiled egg yolk to demonstrate the continuity of the digestive cavity in grafted hydras. Goetsch (1921) offered a drop of egg white to a "hungry" hydra. The hydra opened its mouth but, unable to take in the material, spread the hypostome against the wall of the dish.

Loomis (1955a) states explicitly that hydras can feed only on living members of the Nemathelminthes and higher phyla, ruling out ingestion of algae, mud, bacterial films, and Protozoa, although all of these have been reported.

Zick (1929a) found *H. oligactis* turning green from ingested algae (not symbionts) after their supply of crustacean food has been killed by frost. He observed the hydras gathering and eating the algae, and found that small clumps of algae which he dropped on the tentacles were speedily ingested. The ingestion of both unicellular and filamentous algae by hydras has been observed occasionally in this laboratory.

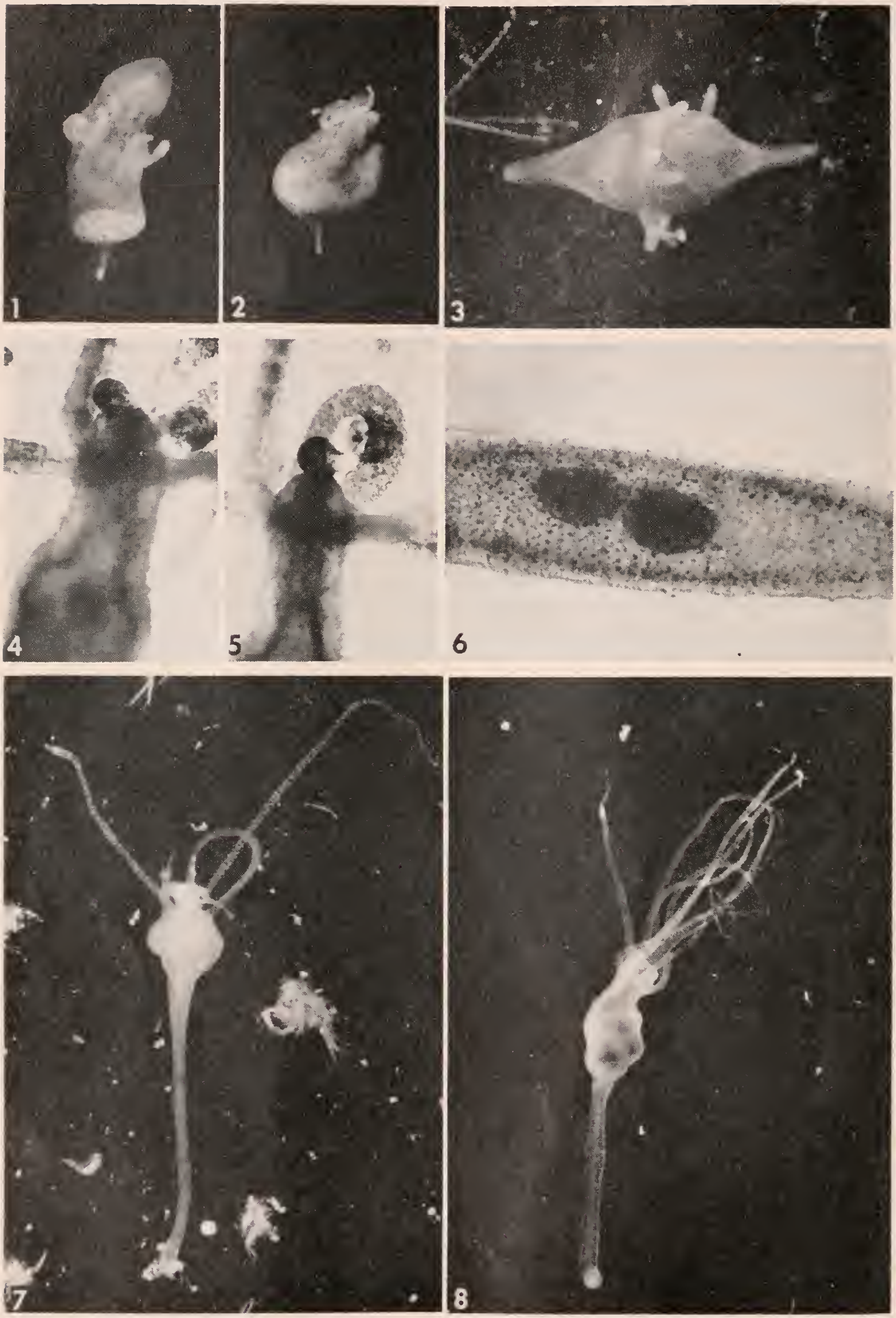
There are several convincing accounts of the ingestion of mud by hydras. The most detailed is by E. B. Wilson (1891), who described a complicated behavior pattern in brown hydras, in which the animals ingested large quantities of mud from the bottom of an aquarium and appeared to be nourished by it. Mud-feeding behavior was also mentioned by Goetsch (1921). Welch and Loomis (1924) published confirmation of the mud-feeding habit and its ability to sustain *H. oligactis* over periods when other foods were scarce, but mentioned that hydras did not always thrive indefinitely on sediment material dredged from the deepest parts of the lake. Haase-Eichler (1931) saw *H. attenuata* conveying bits of mud to the mouth with the tentacles, ingesting it in large quantities, and appearing to thrive.

Goetsch (1921) observed starved hydras spreading the hypostome against a surface film of bacteria and Protozoa, and ingesting this material.

Loomis states (1955a) that hydras are characterized by strict avoidance of cannibalism except in the presence of artificially added GSH, explaining that hydras do not contain sufficient free-flowing intercellular fluids rich in GSH to evoke a feeding reaction. However, there are scattered reports of the swallowing of one hydra by another in the literature, particularly in the case of hungry animals. Goetsch (1921) observed that starved hydras would swallow their own tentacles, feet or buds, although apparently without harm. Kepner and Jester (1927) also observed the swallowing of tentacles in the absence of food objects. Zick (1929a) observed hungry hydras swallowing others of the same species. Hefferan (1901) emphatically stated that *Hydra monoecia* (= *H. oligactis*) would devour *Hydra grisea,* tentacles and all. The ingestion of a member of another species should perhaps be put into a different category, though not necessarily with respect to quantity of intercellular fluid or GSH. In this laboratory, ingestion of one hydra by another of the same species has been observed when the animals were subjected to unusual mechanical or chemical stimulation not involving increased concentrations of GSH. Transfer of hydras to fresh culture fluid—or placing several hydras close together in a small volume of fluid on a clean dry slide or in a clean dry watch glass (transferred with a clean dry pipette)—has often resulted in complete or partial ingestion of one hydra by another.

Hydra pseudoligactis and *H. hymanae* will frequently ingest uninjured *Chlorohydra hadleyi* dropped on the tentacles, when the hydras have not been fed for more than a day, and there is no possibility of contamination with food materials (Fig. 8). In most cases observed, rapid digestion has taken place, with the green color of the disintegrating prey spreading throughout the coelenteron and into buds. A compact mass of green, non-living material is finally ejected. Spontaneous capture has not been observed with these species, but it might easily occur where natural water currents could sweep one hydra into the tentacles of another.

In one instance, regeneration experiments with green and brown hydras (un-



Figs. 1–8.

identified) kept in the same dish were abruptly terminated when the brown hydras ingested the green hydras, and regurgitated only indigestible remains.

The statement that hydras can not feed on animals below the level of the Nemathelminthes (Loomis, 1955a) appears based on scanty evidence. In the case of the excluded phylum Protozoa, Loomis names only two examples as having been tested. Negative results for the first, *Paramecium*, are cited from the work of Ewer (1947), who did not use the same hydra species as Loomis. Positive results obtained by Loomis for the second, *Chaos chaos* (= *Pelomyxa carolinensis*), are dismissed as "the exception that proves the rule."

In this laboratory, the capture and ingestion of *Paramecium* by *Hydra cauliculata* has been observed several times, with a compound microscope. *H. cauliculata* has also been found in nature feeding on small, unidentified ciliates. The ingestion of these ciliates in a freshly collected culture was observed with a dissecting microscope. When enough organic material was provided to sustain the ciliates, the hydras thrived with these as their principal food.

Hydra hymanae has been observed to capture and swallow *Stentor coeruleus* (Figs. 4 and 5). The *Stentor* remain alive within the coelenteron for about ten to fifteen minutes, contracting from time to time (Fig. 6). Shortly thereafter they are killed and apparently digested.

Although Zick (1929b) observed ingestion of captured *Paramecium* in only one instance, McConnell (1929) reported *Paramecium* to be digested normally when placed in the coelenteron of intact hydras.

Concerning Platyhelminthes, Loomis (1955a) tabulates negative results for four varieties tested, two of them quoted from the work of Ewer (1947). However, Baker (1952) states that it is almost certain that the "black Limaces" fed to *H. oligactis* and *H. attenuata* by Trembley (1744) were really the small triclad *Polycclis*, one of the genera found by Ewer to give negative results with *C. viridissima*. In this laboratory, pieces of *Dugesia tigrina*—reported under the name *Planaria maculata* to be rejected by *H. littoralis* (Loomis, 1955a)—and *Dugesia dorotocephala* have been fed to *H. pseudoligactis*, *H. hymanae* and *H. littoralis*. Large *H. pseudoligactis* can eat entire worms, although the worms often escape after the first contact, which sometimes causes swellings on the tentacles of the hydras. Apparently some irritating substance given off in greatest concentration

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FIGURE 1. *Hydra hymanae* swallowing *Dugesia dorotocephala*, four minutes after capturing the worm.

FIGURE 2. The same hydra, twenty minutes later, with the worm folded and completely enclosed within the coelenteron.

FIGURE 3. Two *Hydra hymanae* ten minutes after starting to swallow opposite ends of one *Dugesia*.

FIGURE 4. *H. hymanae* swallowing a captured *Stentor coeruleus*.

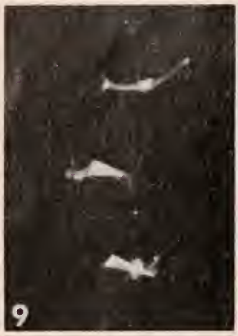
FIGURE 5. The same hydra as in Figure 4, swallowing its second *Stentor*.

FIGURE 6. Middle region of the column of the same hydra with the two *Stentor* inside the coelenteron, ten minutes later.

FIGURE 7. Individual of Loomis' stock of *H. littoralis* feeding on dead *Daphnia* (frozen *Daphnia*, defrosted and spread out at room temperature for three hours).

FIGURE 8. *H. pseudoligactis* swallowing its sixth consecutive *C. hadleyi* in a ten-minute period. Dark objects in the coelenteron are previously ingested hydras. The green hydras were dropped on the tentacles.

In all the examples shown, there was no possibility of contamination with GSH or fresh tissue juice.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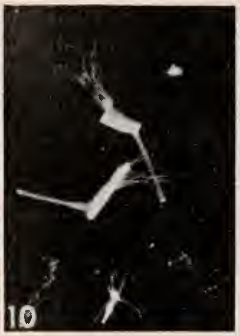
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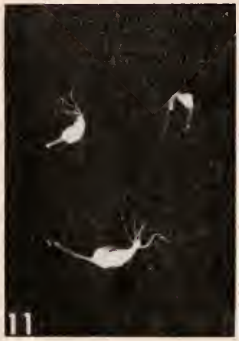
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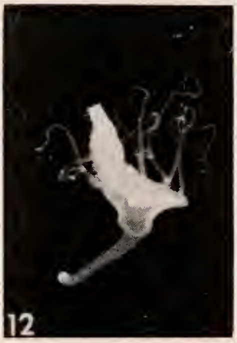
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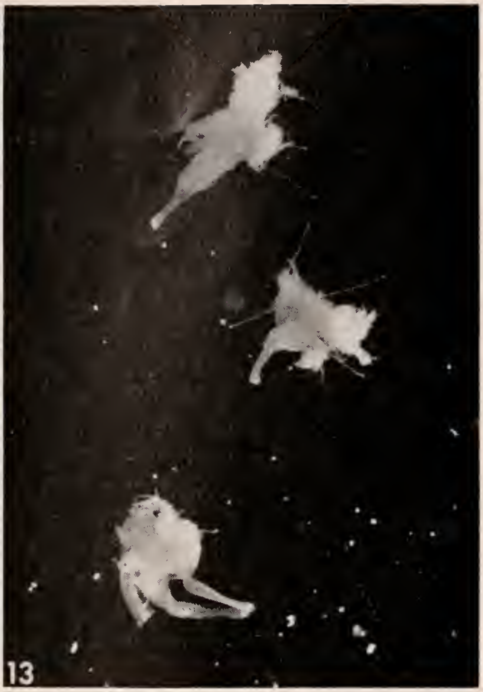
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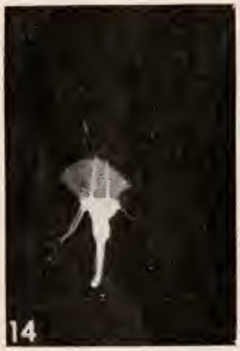
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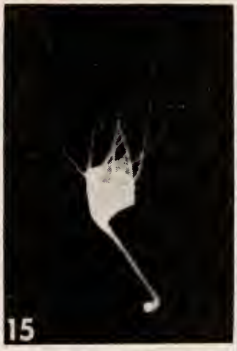
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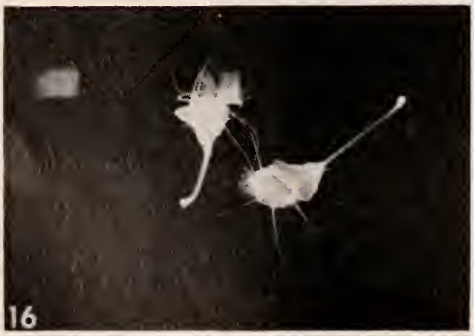
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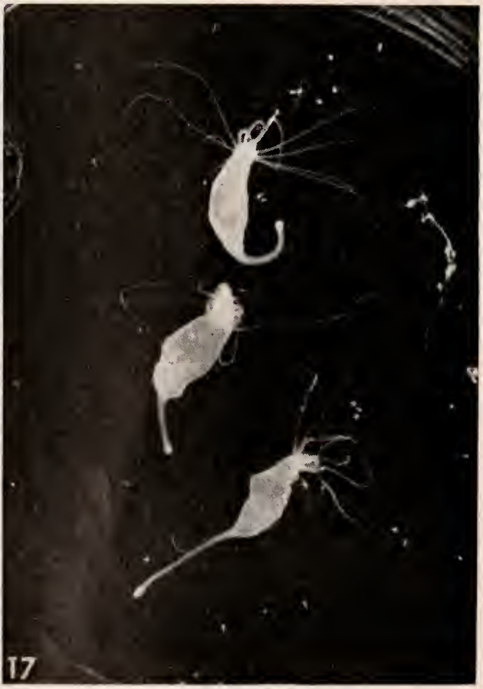
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Figs. 9–17.

near the anterior ends of the worms discourages feeding, but not enough to prevent it in hungry hydras. Posterior halves of worms were taken more readily than anterior halves. Posterior ends allowed to regenerate were taken more readily before the head ends reach normal size and proportions.

Whole planarians dropped into a culture dish with many *H. hymanae* are usually captured and ingested promptly. Sometimes one small hydra will take an entire worm (Figs. 1 and 2). More often two or more hydras take part in subduing a worm and each ingests a portion (Fig. 3). Hydras digest planarians very rapidly, and over the next two or three days gradually develop a peculiar dusky color which lasts for weeks.

The data available are insufficient to support the conclusion that a GSH response could be used by *Hydra* to discriminate between living members of higher and lower phyla. Loomis (1955a) appears to have assumed that any animal not readily ingested by hydras does not contain sufficient fluid rich in GSH, without the necessity for any measurements. When *Chaos chaos (Pelomyxa carolinensis)* was found to be ingested by hydras, Loomis explained that this alone showed that the amoeba had released large quantities of intracellular fluid rich in GSH.

It has not been made sufficiently clear that no one knows how much GSH is present in the surrounding medium when hydras feed normally on their usual prey. No truly accurate quantitative method for estimating GSH exists, since none of the methods for estimating glutathione is completely specific for GSH (Patterson and Lazarow, 1954; Glock, 1961). Estimates published by Loomis (1955a) of amounts of GSH present in the water when hydras are "mixed with living crustacea" were obtained by one of the less specific tests, and give only approximate total SH expressed as GSH. More important, these figures are accompanied by no information concerning the numbers of hydras or crustacea or the volume of fluid in which they were "mixed" to provide the sample. One suspects that the concentrations reported are much higher than those likely to be found in the vicinity of one hydra efficiently ingesting a single *Daphnia,* particu-

FIGURE 9. Three *H. pseudoligactis* after swallowing bits of filter paper flavored with dried *Daphnia* juice that had been treated with hydrogen peroxide. The lowest hydra is disgorging the paper.

FIGURE 10. Two *H. pseudoligactis* and one *H. littoralis* (Loomis' stock), completing ingestion of bits of paper flavored with dried juice of dead *Daphnia.*

FIGURE 11. Three *H. littoralis* (Loomis' stock) after swallowing hard boiled egg white.

FIGURE 12. *H. pseudoligactis,* forty minutes after it was offered a piece of horse meat soaked for one minute in 10^{-5} M GSH. The mouth is loosely open around the food, but swallowing is greatly retarded.

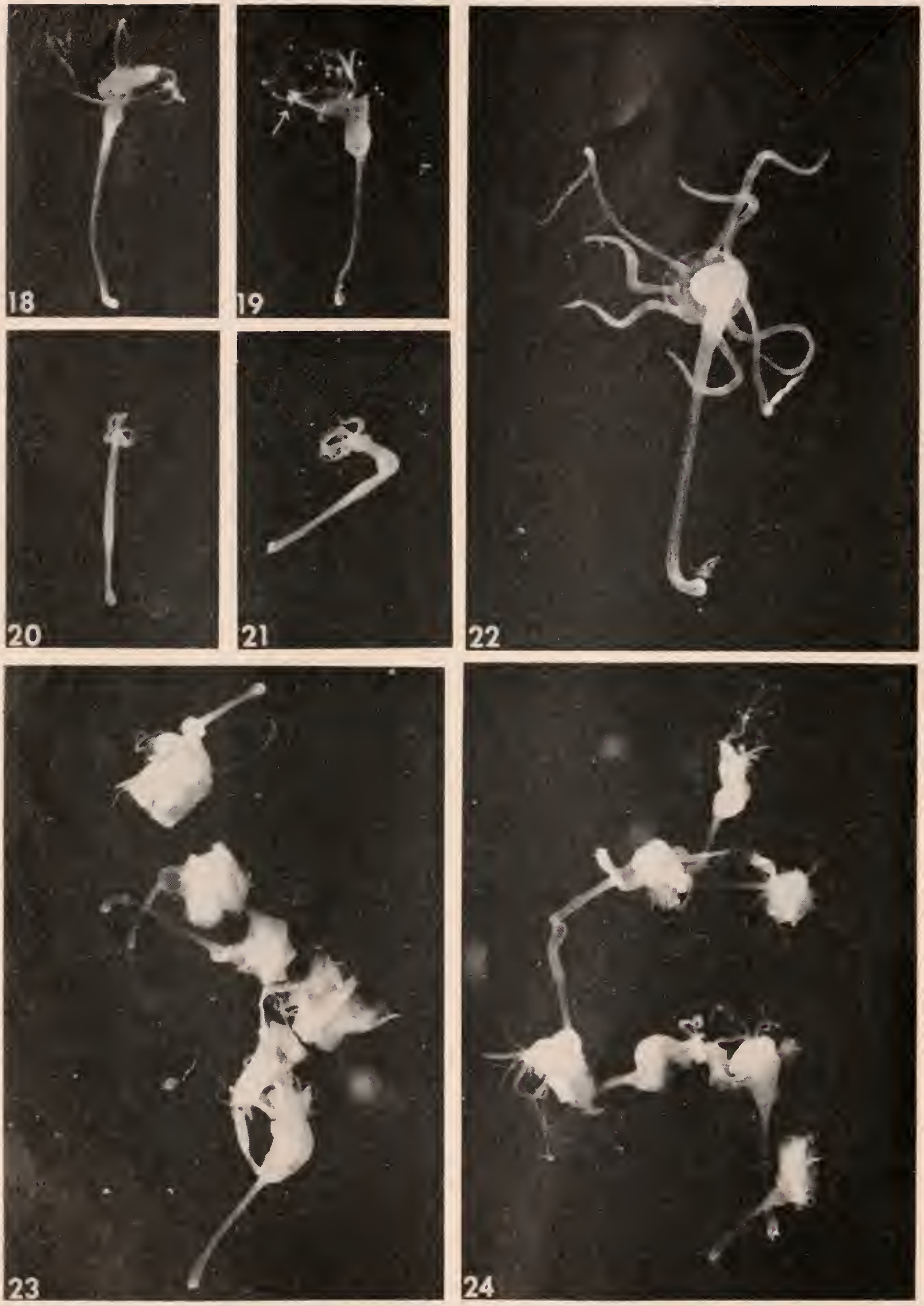
FIGURE 13. Four *H. littoralis* (Loomis' stock) eight minutes after bits of plain horse meat were dropped on the tentacles. Swallowing is nearly half completed.

FIGURE 14. *H. pseudoligactis,* typical appearance of feeding reaction two minutes after block of flavored agar gel was dropped on tentacles.

FIGURE 15. The same hydra eight minutes later. Tentacles are relaxed, the mouth is closely applied to the food object, and swallowing is nearing completion.

FIGURE 16. Two *H. pseudoligactis,* ten minutes after agar blocks containing 10^{-3} M citric acid were dropped on the tentacles. Hydra at right is bringing rim of mouth up around translucent agar block.

FIGURE 17. Three *H. pseudoligactis* fifteen minutes after being offered agar blocks containing 10^{-2} M urea.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Figs 18–24.

larly in the case of *H. littoralis*, an inhabitant of swiftly moving water. Hydras immersed in solutions of 10^{-2} to 10^{-5} *M* GSH, commonly used to demonstrate prolonged mouth opening, are certainly exposed to unnaturally large amounts of this substance. The juice of homogenized *Artemia,* shown by Loomis (1955a) to produce responses like those shown to GSH solutions, might be expected to contain far larger amounts of GSH than a hydra could release from its prey. The minute size of the holes made in the prey by the stenoteles of hydras may be estimated from the figures given by Jennings (1906) or from the electron micrographs of Semal-Van Gansen (1954), who found the stenotele thread about one micron in diameter. The nematocysts themselves largely occlude these openings. The punctures may be few, and not all of them reach juicy areas, so the amount of fluid released must often be extremely small. Loomis (1955a) seems to be the first to claim observation of fluid leaking from these small punctures (under a magnification of 500 ×).

Among the most telling arguments against a hormone-like specificity to GSH for feeding are reports of the ingestion by hydras of filter paper dipped in solutions lacking in GSH. The earliest is that of Wagner (1905), who found that hydras starved for one week readily swallowed filter paper dipped in beef tea, prepared with beef extract, but would not swallow filter paper soaked in plain water. Hydras transferred bodily to beef tea showed no reaction unless offered the end of a glass rod to swallow, showing the necessity for both chemical and mechanical stimulation in feeding. Wagner demonstrated that the mechanical factor declines in importance with increasing need for food, by starving hydras twelve days and then transferring them to beef tea. Their tentacles writhed, their mouths opened momentarily, and their bodies bulged as though they had swallowed a drop of the flavored medium.

Wagner (1905) also reported that hydras immersed in quinine solutions gave "feeding reactions" like those in beef tea, but finally perished with mouths widely opened. It has been stated (Lenhoff and Schneiderman, 1959; Lenhoff, 1961a) that some "deleterious substances" can cause mouth opening in hydras but cannot induce ingestion of "inert objects" or "attempts to swallow the walls of the dish" shown by hydras exposed to GSH. In this laboratory it has been found that very dilute solutions of quinine hydrochloride, 10^{-4} to 10^{-6} *M*, can produce pro-

FIGURE 18. *H. pseudoligactis* with open mouth and partly contracted tentacles (a very long tentacled species) starting to swallow plain washed filter paper under the influence of 5×10^{-6} *M* quinine hydrochloride.

FIGURE 19. *H. pseudoligactis*, with tentacles contracted and mouth open, swallowing *H. littoralis* (hypostome indicated by arrow) under the influence of 5×10^{-6} *M* quinine hydrochloride.

FIGURES 20 and 21. *H. littoralis*, showing peculiar curved, crescentic outline of tentacles contracted under the influence of 10^{-5} *M* GSH.

FIGURE 22. *H. pseudoligactis* with mouth open, spreading hypostome against floor of dish, under the influence of 5×10^{-6} *M* quinine hydrochloride.

FIGURE 23. Six *H. littoralis,* one-half hour after being offered bits of horse meat dipped in 10^{-5} *M* GSH. Tentacles are contracted and mouths are widely opened, but swallowing is retarded. Only in the hydra near the bottom of the picture has the food entered the main body of the column.

FIGURE 24. Seven *H. littoralis* fifteen minutes after being offered bits of plain horse meat. Tentacles are more relaxed, the food is well down inside the column in most instances, and swallowing is nearing completion in some.

longed mouth opening, writhing and curling of tentacles, spreading of the hypostome against the substrate (Fig. 22), ingestion of other hydras (Fig. 19), and ingestion of "inert objects" such as plain washed filter paper (Fig. 18). Thus dilute quinine solutions can be said to "induce feeding" like dilute GSH solutions. In a concentration of 5×10^{-6} M, quinine hydrochloride can be tolerated by hydras for several hours without apparent harm.

Balke and Steiner (1959) published an interesting report of the ingestion by *H. oligactis* of bits of filter paper dipped in dilute solutions of pure chemicals, including lactic acid, ascorbic acid, acetic acid, sodium acetate, and glutamic acid, as well as GSH. Over 95% of responses were positive to lactic acid at a concentration somewhat less than 10^{-2} M. Plain filter paper was ingested in 6% to 12% of trials.

Hydras in this laboratory offered bits of filter paper dipped in solutions of 10^{-2} M and 10^{-3} M lactic acid ingested them promptly in a high percentage of cases, confirming the work of Balke and Steiner. However, dilute solutions of lactic acid did not induce the abnormally prolonged mouth opening or peculiar crescentic curling of tentacles seen with solutions of GSH (Figs. 20 and 21).

Altogether a tremendous weight of evidence from the literature and from personal observation opposes the report that hydras are confined to living prey of higher animal phyla by exclusive dependence on GSH for feeding. Experimental work was undertaken to explore possible grounds for the discrepancy.

Experimental Evidence

Feeding hydras on meat

Since statements that hydras will not eat meat (Loomis, 1955a; Loomis and Lenhoff, 1956) were associated with experiments using *H. littoralis*, and since none of the published reports of successful meat feeding dealt with this species, a comparative study of responses to meat was undertaken using *Chlorohydra hadleyi, Hydra americana, H. carnea, H. cauliculata, H. hymanae, H. oligactis, H. pseudoligactis,* two as yet undescribed species, locally collected *H. littoralis*, and a clone descended from Loomis' stock of *H. littoralis* (obtained through the courtesy of Dr. Helen D. Park). All of the stocks had been maintained in uncrowded fingerbowls at 18–20° C., and fed daily on washed *Artemia* nauplii for several weeks. Eleven fingerbowls were used for testing, each containing ten large individuals of one hydra stock, in 200 ml. of filtered pond water. Each day for ten days, a small piece of raw chopped horse meat, purchased as pet food, was dropped on the tentacles of each hydra, with clean watchmaker's forceps. The pieces were about 0.5 to 2.0 mm. in diameter, depending on the size of the hydras. After one half hour, the number of pieces ingested in each dish was noted, and the remaining pieces removed. An hour later, the water was changed, and the animals left undisturbed until the next feeding. After ten days the hydras were compared with matched, unfed controls, and with animals fed on *Artemia* daily.

Hydras of the eleven stocks ingested horse meat the following percentages of times offered: *H. americana*, 99%; *H. pseudoligactis*, 98%; *H. hymanae*, 97%; two undescribed species, 96% and 95%; *H. cauliculata*, 92%; *H. oligactis*, 90%;

H. carnea, 87% ; locally collected *H. littoralis*, 78% ; Loomis' stock of *H. littoralis*, 64% ; *C. hadleyi*, 32%. Negative responses were most numerous in the first few days. Feeding responses resembled those seen with living prey of comparable size (Figs. 13 and 24). Swallowing was completed in five to twenty-five minutes. The food was retained for several hours, after which colorless, glutinous remnants were ejected.

At the end of ten days, the animals of all eight stocks were obviously larger than the unfed controls, and at least one new bud had been formed in each dish. All were paler in color and had formed fewer buds than the animals fed daily on *Artemia*. It was concluded that all of the species tested showed normal feeding responses to horse meat and obtained nourishment from it, but that one bit of horse meat a day was insufficient to support good growth.

For the next five days, the same animals were fed on bits of chopped beef, with closely similar results.

Reactions to dried meat juice

Although GSH is believed to be rapidly oxidized in tissues after death, an experiment was planned to give additional opportunity for oxidation in a thin film of dried meat juice. A clean, straight, glass-headed dressmaker's pin was pushed into a piece of beef and withdrawn covered with meat juice. The pin was held submerged in culture fluid just above a hydra until the hydra's mouth opened, and then lowered to permit the hydra to swallow the end of the pin—as in an experiment by Pickens (1933). In five trials with *H. pseudoligactis*, the hydras swallowed the pins each time. *H. littoralis* responded with writhing of tentacles and brief mouth opening, and with partial ingestion in three cases out of five, but no complete swallowing (partly because of mechanical difficulties associated with its smaller size).

Ten pins were plunged into beef chunks, withdrawn coated with meat juice, and allowed to dry in the air at room temperature overnight. The next day these pins produced responses in *H. pseudoligactis* and *H. littoralis* similar to those provoked by pins freshly withdrawn from meat.

Feeding hydras on dead Daphnia

Loomis (1955a) did not publish details of his unsuccessful attempts to induce hydras to feed on non-living materials, except to state that dead *Daphnia* agitated vigorously among the tentacles were not ingested by his hydras, and to caution that in repeating this test one should take care to use only *Daphnia* that had been dead for several hours and that were uncontaminated with traces of fresh tissue juice.

In the expectation that long dead *Daphnia* might prove unacceptable to hydras, attempts were made to feed these to several species. Fingerbowls were prepared as before, each with ten hydras of each of the eleven stocks already mentioned. A package of frozen *Daphnia* was purchased from a pet shop, and a portion defrosted, spread out thinly in a clean Petri dish, and allowed to stand loosely covered at room temperature for three hours. A small portion of dead *Daphnia* was picked up with forceps and dropped on the tentacles of each hydra. Within half an hour at least one *Daphnia* had been ingested by one to eight hydras in each dish. One hour later, the water was changed.

The defrosted *Daphnia* were then uncovered and allowed to dry for ten hours to a gummy mass, bits of which were offered to the same hydras. A portion of this material was ingested by the following numbers of hydras in each dish: *H. pseudoligactis,* 10; *H. hymanae,* 10; two undescribed species, 9 each; *H. americana* and *H. cauliculata,* 8 each; *H. carnea,* 7; *H. oligactis,* 6; *H. littoralis,* 5; Loomis' stock of *H. littoralis,* 4; *C. hadleyi,* 2. The material had not lost its activity. It may have gained effectiveness by becoming more concentrated on drying.

To investigate the possibility of maintaining hydras on a diet of dead *Daphnia,* the culture dishes of *H. pseudoligactis, H. hymanae,* and *H. littoralis* (Loomis' stock) were offered dead *Daphnia* for a two-hour period once a day for two additional weeks. Defrosted *Daphnia* spread out at room temperature for two hours were dropped on the tentacles of each hydra. The water was changed at the end of each feeding period. The hydras received no other food. After the first three days, almost every hydra ingested several *Daphnia* at a feeding (Fig. 7). At the end of two weeks, some new buds had appeared in each dish, and the hydras were larger, stouter, and with distinctly longer tentacles than similar animals fed on *Artemia,* although the *Artemia*-fed animals had formed many more buds. Agitation was not necessary to induce feeding.

Loomis' negative results may have been due in part to unduly vigorous agitation of the dead *Daphnia;* to chemical changes in the long dead *Daphnia* rendering them repellent; to a reduced responsiveness in his animals resulting from excessive handling or rinsing in buffers or other solutions just before testing; or to poor physiological condition of his animals.

Hydras grown in mass culture by the methods of Loomis and Lenhoff (1956) appear to be inferior in size and activity to freshly collected specimens or those cultured under conditions more closely approaching the natural. A high rate of asexual reproduction does not necessarily signify optimum condition in all respects. Dr. Libbie H. Hyman reports (personal communication) that the hydras she observed in Loomis' laboratory showed a shortened column and tentacles and appeared to be in a state of mild depression. Such animals may not exhibit normal feeding responses. Although convenient for laboratory use, *Artemia* nauplii are not a natural food for hydras. Prolonged maintenance of cultures on this diet alone may foster abnormalities such as the stunted form and protracted sexual periods found in Loomis' stock of *H. littoralis.* Hydras kept in solutions containing Versene may fail to show normal feeding responses, according to observations in this laboratory, partly supported by observations of Lenhoff and Bovaird (1959).

Ingestion of filter paper containing dried Daphnia juice

A portion of frozen *Daphnia* was homogenized and centrifuged. The supernatant was mixed with an equal quantity of distilled, de-ionized water, filtered, and taken up on washed filter paper and dried. Small bits (about 0.5 by 1.5 mm.) of this paper were dropped on the tentacles of five *H. pseudoligactis,* five *H. hymanae,* and five *H. littoralis.* All of the animals grasped the paper with the tentacles, and opened their mouths against it, but some later released it. They appeared to have some difficulty with the stiff, bristly surface. The paper was completely ingested by all five of the *H. pseudoligactis,* three of the *H. hymanae,* and one of the *H. littoralis* (Fig. 10).

Loomis (1955a) had reported that treatment with hydrogen peroxide caused juice from homogenized *Artemia* to lose its effectiveness in inducing feeding in hydras, so trials were made here with peroxide-treated juice from frozen *Daphnia.* (Comparison is difficult because Loomis gauged effectiveness of *Artemia* juice by its ability to induce ingestion of dead *Daphnia,* which have been shown here to be ingested without treatment.)

About two ml. of frozen *Daphnia* were mixed with an equal quantity of one per cent hydrogen peroxide, homogenized and centrifuged. The filtered supernatant was taken up on filter paper, bits of which were dropped on the tentacles of five *H. pseudoligactis,* and five *H. littoralis.* As had been expected, the presence of excess peroxide was irritating, and produced strong contraction of column and tentacles in all hydras, with no feeding responses. Bits of washed filter paper dipped in the filtrate were then air dried at room temperature. After five days they were dropped on the tentacles of five *H. pseudoligactis* (unfed for one day). Ingestion took place in three cases (Fig. 9).

Feeding hydras on hard boiled egg

The failure of hydras to ingest egg, asserted by Loomis and Lenhoff (1956), would be expected to follow exclusive dependence on GSH for feeding. Data for GSH content of hard boiled hens' eggs could not be found, but figures quoted by Glock (1961) in the Biochemist's Handbook give the negligible value for whole raw egg of 1.16 mg. per 100 grams wet weight, concentrated almost entirely in the yolk. Any GSH present in hard boiled eggs would not appear in solution in juices readily released on stenotele puncture.

Hens' eggs from a supermarket were boiled for ten minutes and cooled to room temperature. Fresh hydras of three species were arranged in fingerbowls as before, and a piece of hard boiled egg white dropped on the tentacles of each, once a day for five days. *H. pseudoligactis* ingested egg white 98% of the times offered; *H. hymanae,* 86%; *H. littoralis* (Loomis' stock), 62% (Fig. 11). The egg white was retained for 12 to 20 hours, and was not completely dissolved within the coelenteron. A mucilaginous mass was finally ejected. After five days on a diet of egg white, the hydras were pale and had decreased slightly in size, but appeared larger and stouter than unfed animals.

Different individuals of the same three species were tested separately for ingestion of hard boiled egg yolk. Bits of yolk dropped on or near the tentacles spread into piles of small globules which were gathered up by the tentacles and ingested. *H. pseudoligactis* fed on this 100% of the times offered; *H. hymanae,* 98%; and *H. littoralis* (Loomis' stock), 78%. Hydras fed on egg yolk were larger than those fed egg white, but formed no buds in the five-day test period. They appeared yellowish, especially *H. pseudoligactis.* The egg yolk was retained for twelve hours or more, and then ejected in a white, compact, sausage-shaped mass.

In additional tests, egg yolk was left in a culture dish for several hours. The hydras continued to ingest particles of yolk intermittently, becoming distended until they were almost globular. When bits of egg yolk were dropped into a dish at some distance from any hydras, several hours later some of the hydras were found to have ingested some of it, having come in contact with it either by chance or guided by a chemical sense.

Feeding hydras bits of filter paper dipped in dilute solutions of various chemicals

Bits of washed filter paper dipped in dilute solutions of nicotinic acid, citric acid, riboflavin, urea, or sodium chloride were dropped on the tentacles of hydras of all eleven stocks previously mentioned, following the method of Balke and Steiner (1959). Some positive responses were obtained from all stocks with each chemical.

Feeding hydras bits of agar containing dilute solutions of various chemicals

For a more complete investigation of the effectiveness of the compounds named above, a substitute for washed filter paper as a vehicle was sought. The paper seemed undesirably rough and angular, and tests with colored substances showed that solutions diffused out of it very rapidly. It was found convenient to incorporate the solutions in a 1.5% agar gel. Agar could easily be offered in smooth, solid portions of any desired size and shape, furnishing a mechanical stimulus with a more slowly diffusing concentration gradient, and permitting crucial judgment of a true feeding reaction: the seizing, conveying to the mouth and ingestion of solid objects.

Agar itself, as an extract of various seaweeds, varies in composition from different sources. However, its chemical composition has been scrupulously and repeatedly analyzed (Araki, 1959) and it can safely be declared not to contain even trace amounts of GSH or the proteases more recently found by Lenhoff and Bovaird (1960) to induce "hormone-like responses" in hydras. Loomis and Lenhoff (1956) have unequivocally stated that hydras will not ingest bits of agar gel unless these

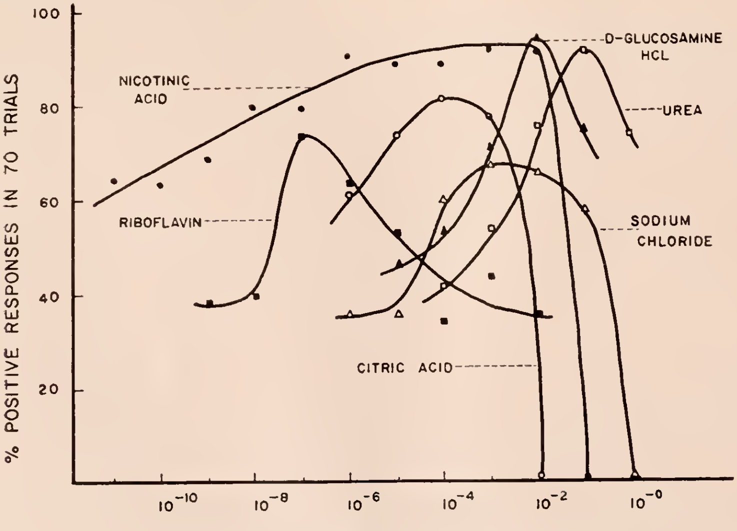

FIGURE 25.

are first dipped in solutions of GSH. Like the filter paper used here and by Balke and Steiner to demonstrate the effectiveness of pure solutions in inducing feeding reactions, agar should come much closer to qualifying as an "inert object" to test feeding than could the dead *Daphnia* used by Loomis (1955a) to test the activity of tissue juice that had been treated in various ways.

Since all the hydra species tested had given positive feeding responses to the same substances, a form could be chosen on the basis of convenience in handling. *H. pseudoligactis* was selected because it was the largest and one of the most responsive, the easiest to handle and to observe.

The *H. pseudoligactis* used were members of a single clone, without buds or gonads. For several days before testing they were kept ten to a dish of 150 ml. of filtered pond water, at 18–20° C., and fed once a day with *Artemia* nauplii. They were tested 12 to 20 hours after their last feeding. Agar gel was cut into cubes about 2 mm. on each side, and a cube dropped onto the tentacles of each of ten hydras in a dish. Feeding usually began in a few seconds (Fig. 14). Ten minutes later the number of positive responses was recorded. Responses were judged positive only for hydras with mouths widely opened and firmly surrounding the partly ingested agar (Fig. 15).

Tests of dilutions of riboflavin and D-glucosamine HCl were run concurrently as one series; urea, citric acid, and sodium chloride as another series; and nicotinic acid as a third series. Plain agar gels prepared with filtered pond water and with distilled, de-ionized water were used as controls. Positive responses to plain agar ranged from 0 to 90% on different days, possibly representing responses to small amounts of pyruvic acid or sulphate, present in agar (Araki, 1959). The variation in sensitivity was apparently associated with alterations in the physiological condition of the animals, since it was reflected in their responsiveness to all solutions tested at the same time. It was not related to a few hours' difference in time since the last feeding, but may have resulted from cyclic fluctuations in metabolism. Hydras and their relatives have long been known to show great variation in chemical sensitivity (Wagner, 1905). Pantin (1942) found considerable differences in sensitivity between normal tentacles on an anemone. To compensate for these variations, each dilution was tested on seven different days. Slightly less than 40% of the hydras tested responded positively to plain agar in each series. This was interpreted to show an averaging out of differences in sensitivity, to give roughly comparable results in the three series.

The results of the tests are summarized in Figure 25. There was a peak response to a particular concentration of each substance, and in some cases a sharp change to a negative response at higher concentrations. Nicotinic acid, with a peak effectiveness of 93% at 10^{-3} *M*, is notable for its high effectiveness over a wide range of concentrations and in extreme dilution, exceeding in these respects the reported potency of GSH, tested with filter paper (Balke and Steiner, 1959). Urea (92% at 10^{-1} *M*), and D-glucosamine HCl (95% at 10^{-2} *M*) were about equally effective, but over a narrow range. Riboflavin showed a peak effectiveness of 74% at the very low concentration of 10^{-7} *M*. Citric acid gave positive responses in 82% of cases at 10^{-4} *M*. Sodium chloride had a peak effectiveness of 68% at 10^{-3} *M*, exceeding 60% over the 10^{-2} to 10^{-4} *M* range.

It seems likely that diffusing substances that induce ingestion of bits of paper or agar gel might have synergistic effects when they occur together in natural foods.

Feeding hydras meat and egg white dipped in GSH

Ten *H. pseudoligactis* and ten *H. littoralis* were offered bits of hard boiled egg white soaked for one minute in 10^{-5} *M* GSH. The experiment was repeated with bits of horse meat. In every instance, GSH greatly retarded the swallowing process, although it caused prompt tentacle contraction and mouth opening (Figs. 12 and 23). Superficially the animals appeared to be feeding, but their mouths hung loosely open about the food for half an hour or more, and swallowing was not always completed. The *H. pseudoligactis* tested swallowed plain egg white and horse meat every time, in ten to twenty minutes. With GSH, all mouths were still wide open after thirty minutes, and swallowing was not completed with five pieces of egg white and four of horse meat. The *H. littoralis* swallowed the plain egg white in six cases, and the horse meat in seven, completing swallowing in ten to twenty minutes. Egg white with GSH was completely ingested in nine cases, horse meat in six, with forty minutes the minimum time for complete swallowing.

Loomis (1955a) reported that GSH solutions interfered with feeding on *Daphnia* by hydras. He explained that in the presence of GSH in the surrounding medium the hydras become "confused," having no gradient to follow, hence they fail to locate the prey and their coordination in feeding is impaired. This explanation is difficult to reconcile with the accompanying statements that the same solutions induced hydras to ingest other hydras and to "attempt to swallow the walls of the dish," which could not have furnished a GSH gradient. It can not explain interference with swallowing of egg or horse meat soaked in GSH, since these could furnish a gradient.

The prolonged mouth opening induced by GSH hinders migration of the rim of the mouth over the surface of the food to complete swallowing. GSH solutions also produce peculiar, sustained contraction of the tentacles, well shown in photographs by Loomis (1955a) and in Figures 20 and 21 of this paper. This posture is shown in modified form in drawings by Lenhoff (1961a, 1961b). It differs markedly from the attitudes of normally feeding animals (Figs. 4, 5, 7, 8, 13). Descriptions of normal feeding in hydras have emphasized that after the tentacles have conveyed the food to the mouth they relax and ignore the prey (Hartog, 1880; Wagner, 1905), taking no further part in feeding except to gather more food or to reach up momentarily around the food as if measuring the amount yet to be encompassed. Normal feeding in *H. littoralis* is shown in the excellent photographs by Tice (Buchsbaum, 1938). Hydras exposed to GSH may stand for half an hour with mouths loosely opened about a food object without ever ingesting it (Fig. 12). It seems unreasonable to insist that this duplicates normal feeding behavior.

It has been stated that only ingestion of "inert objects" should be taken as proof of the ability of chemical substances to induce feeding in hydras (Lenhoff, 1961a, 1961b). However, the only objects reported by the same author and by Loomis (1955a) to be ingested by hydras under the influence of GSH are: dead *Daphnia*, meat, agar, egg, other hydras, and "the walls of the dish." The criterion actually used to demonstrate effectiveness of GSH in inducing "feeding responses" has been prolonged mouth opening. In a table showing the effect of starvation on the sensitivity of hydras to GSH (Loomis, 1955a), one-third of the positive responses tabulated are based on a reaction of tentacle writhing alone; and the remaining two-thirds on tentacle writhing and mouth opening, with ingestion not mentioned. In a paper by Lenhoff and Bovaird (1959) concerning the requirements of "surface GSH receptors" in hydras, effectiveness in producing feeding responses is measured by the average length of time the hydras' mouths are held open—ranging from 21.2 to 28.5 minutes in the presence of 10^{-5} M GSH, except when the hydras were deprived of calcium. Effects of calcium deprivation are attributed to effects on hypothetical receptors, without consideration of the alternate possibility of effects on contractile elements.

Increasing sensitivity to GSH with starvation may be explained by the fact that starved hydras are increasingly sensitive to any stimulation, and even take up vital stains more readily, suggesting an increased permeability (Spangenberg and Ham, 1960).

In this report, responses termed "feeding reactions" have always involved the following: grasping of solid objects by the tentacles, accompanied by nematocyst discharge; conveying of the objects to the mouth by bending (sometimes with contraction) of one or more tentacles; momentary mouth opening to receive the food; relaxation of the tentacles after the food has reached the mouth; gliding of the rim of the mouth rapidly over the food to encompass it—in a characteristic manner that has been likened to the action of an "automatic stocking" or to the way in which a snake swallows its prey (Hartog, 1880; Wagner, 1905); tight closure of the mouth over the food as soon as it has been surrounded; circular contraction of the distal portion of the column, forcing the food down into the middle region of the animal; retention of the food object for one-half to several hours; eventual rejection of the indigestible material. It is difficult to see how this sequence of events could be declared anything but a true feeding reaction, but it has been observed repeatedly when GSH could not have provided the stimulus.

I should like to express my deep gratitude to Dr. Libbie H. Hyman for reviewing this manuscript, and for most helpful suggestions; and to Dr. George K. Reid for his advice and encouragement in a portion of this work.

SUMMARY AND CONCLUSIONS

1. Of ten North American hydra species tested, none has been found restricted to a diet of living animals of higher phyla, or exclusively dependent on release of GSH in juices of prey for initiation and coordination of feeding.

2. Hydras have been observed and photographed swallowing Protozoa, flatworms, other hydras, dead *Daphnia*, bits of beef and horse meat, pieces of hard boiled egg white and yolk, and bits of washed filter paper and of agar gel containing dilute solutions of various chemicals—all in the absence of artificially added GSH or fresh tissue juice. The chemicals used here to induce feeding cannot be considered deleterious in the concentrations and quantities employed.

3. All of the hydras tested responded to the same substances, but some species seemed more sensitive to chemical stimulation than others. The results of an investigation dealing with one species should not be extended to others without additional information.

4. Recent reports of tests purporting to demonstrate the effectiveness of GSH in inducing feeding have dealt almost exclusively with the ability of GSH to induce prolonged mouth opening, although these same reports have emphasized that only

the ability to induce ingestion of "inert objects" can prove the effectiveness of chemicals in inducing feeding.

5. Hydras have been observed and photographed opening their mouths widely, spreading the hypostome against the substrate, and swallowing washed filter paper and other hydras under the influence of 5×10^{-6} *M* quinine hydrochloride.

6. Of the chemicals tested that induced ingestion of solid objects, only GSH and quinine hydrochloride regularly produced prolonged mouth opening in the absence of a mechanical stimulus. This does not occur in normal feeding. The ability of compounds to initiate or coordinate feeding does not depend on their ability to induce prolonged mouth opening, which does not represent a true feeding response.

7. Dilute GSH solutions (10^{-2} to 10^{-5} *M*) retard and interfere with normal swallowing. Hydras exposed to these solutions show a peculiar and abnormal contraction of the tentacles.

8. Concentrations of GSH appearing in the surrounding medium when hydras capture their normal prey are probably much lower than the concentrations commonly used to demonstrate the prolonged mouth opening that has been loosely termed a "specific feeding reaction." In lower concentrations GSH may function as one of a number of chemical substances active in controlling feeding in hydras. In higher concentrations it may be mildly toxic, producing the striking effects that have been so well publicized.

9. A detailed investigation of the action of GSH in producing prolonged mouth opening in hydras is still of considerable interest, as would be a study of the effects of quinine hydrochloride. However, the hydras' lives do not depend on this reaction, as had been claimed, since feeding can be regulated by other means.

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CARDIAC RESPONSES OF FISHES IN ASPHYXIC ENVIRONMENTS[1]

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Many lung-breathing animals are adapted to an aquatic life. Whales, seals, penguins, and crocodiles are representative. Whenever these animals dive and swim submerged, they show marked physiological adjustments (Scholander, 1940; Andersen, 1961). Even though a pronounced bradycardia occurs, a nearly normal blood pressure is maintained. In addition, lactic acid builds up in the muscles and is flushed into the circulation when the animal surfaces and breathes. Analogous to these diving animals are certain species of fish that subject themselves to air. Some fish migrate over land, others spawn out of water, and a few take to the air in order to escape predators. Such animals as the hagfish also encounter asphyxic situations when they bury in the bodies of other vertebrates.

The question arises—do fishes that naturally venture into air exhibit compensatory physiological changes analogous to those found for lung-breathing animals? In the present work a few fishes that subject themselves to an asphyxic condition have been investigated—the beach-spawning grunion, the California flying fish, an air-dwelling mudskipper, and one of the scavenging hagfishes. Since heart frequency is a conspicuous manifestation of an asphyxial response, this parameter has been studied.

METHODS

A portable Sanborn Visette electrocardiograph, model 300, proved adaptable for field work. This direct-writing instrument was powered by a Heathkit converter, model PC 1, operating from a storage battery.

Electrodes were made from number 28 stranded plastic-covered wire (Fig. 1). Two slits, 1–3 cm. apart, made in the insulation allow for exposure and removal of the wire between them, leaving a wire electrode protruding from each opening. The fish remains in water as electrodes are threaded through the body wall near the heart (Fig. 1). This method has proved practical in use on teleosts, sharks, rays, and even on writhing mucus-secreting hagfish.

An asphyxial condition was induced by various means. Containers were used to provide alternate flooding and draining for the grunion and the hagfish. The flying fish was lifted from water on a horizontal net, while a wire-mesh enclosure served for the mudskipper. Most of the teleosts and sharks studied remain calm during transitions from water to air, when supported by such a shallow net.

DESCRIPTION AND RESULTS

GRUNION (*Leuresthes tenuis*)

In the coastal waters of southern California lives one of the few fish species that lays eggs on land (Walker, 1952). The spawning process occurs on evenings

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[1] Contributions from Scripps Institution of Oceanography, New Series.

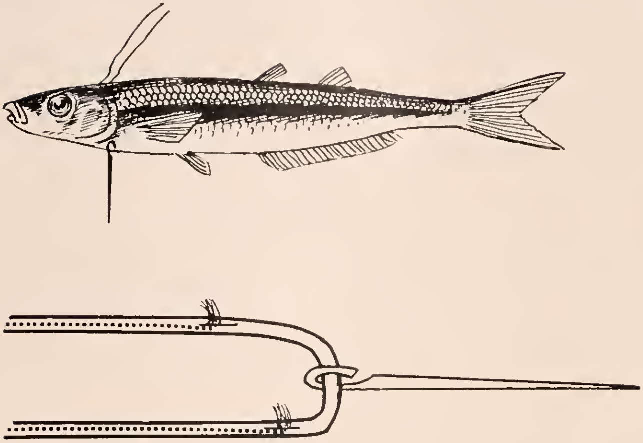

FIGURE 1. (Top) Grunion with inserted electrodes. (Bottom) Enlarged diagram of the electrodes.

following highest spring tides when the grunion ride onto the beach on large waves. As a wave recedes, they swim vigorously, so as to strand themselves high on the beach. By twisting the body the female digs tail-first into the sand, and then lays eggs that are fertilized by nearby males. (These eggs remain undisturbed by water until inundation by high tides about two weeks later causes them to hatch.) Freeing herself from the sand, the spent fish takes a few flops toward the water, or lies until a high wave takes her to sea. A total time of approximately one minute elapses while the grunion's gill chambers are out of contact with water. At times, however, a fish will be stranded out of water's reach for several minutes. This exposure it tolerates well.

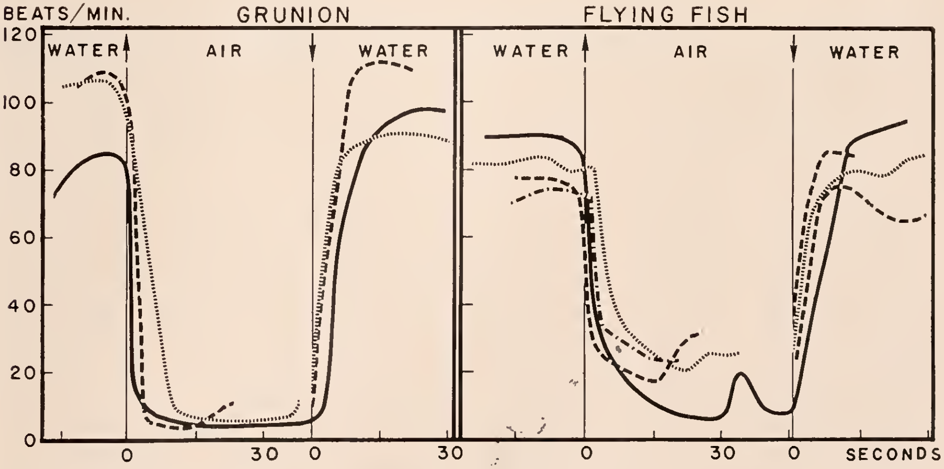

FIGURE 2. Heart rates of grunion and flying fish before, during, and following air exposure. Air exposures terminate at different times but recoveries are synchronized.

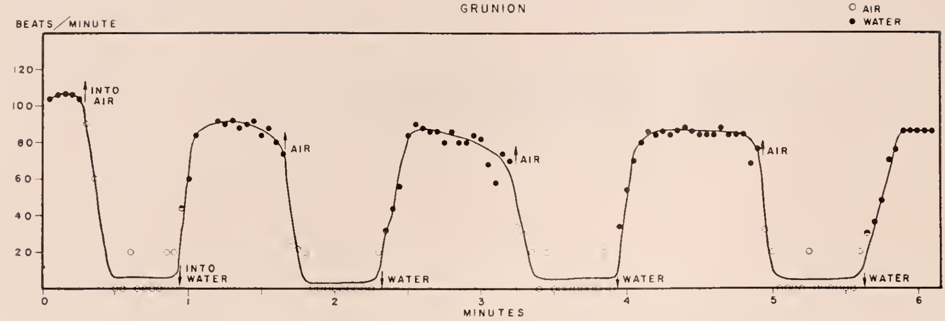

Figure 3. Repetitive exposures of a grunion to air. Observations are at three-second intervals.

Grunion, when taken out of water, immediately develop an extreme bradycardia (Fig. 2). The heart rates often slow from more than 100 to fewer than 10 beats per minute. The original, or slightly higher, pace is quickly resumed when the fish are returned to water (Figs. 2, 3). However, prolonged exposures of 5–10 minutes produce slow cardiac recovery. These laboratory results were confirmed by records taken on the beach during spawning.

CALIFORNIA FLYING FISH (*Cypselurus californicus*)

Flying fishes are pelagic plankton feeders that take to the air when pursued by predators. Their flight of as much as 200 yards is initiated by an accelerated take-off ("taxi") and continues as a passive glide. Compound flights, consisting of intermittent glides and tail accelerations, may keep the fish exposed to air for relatively long periods. Hubbs (1933, 1937) observed that the simple flights average about two seconds, although he noted single flights lasting as long as

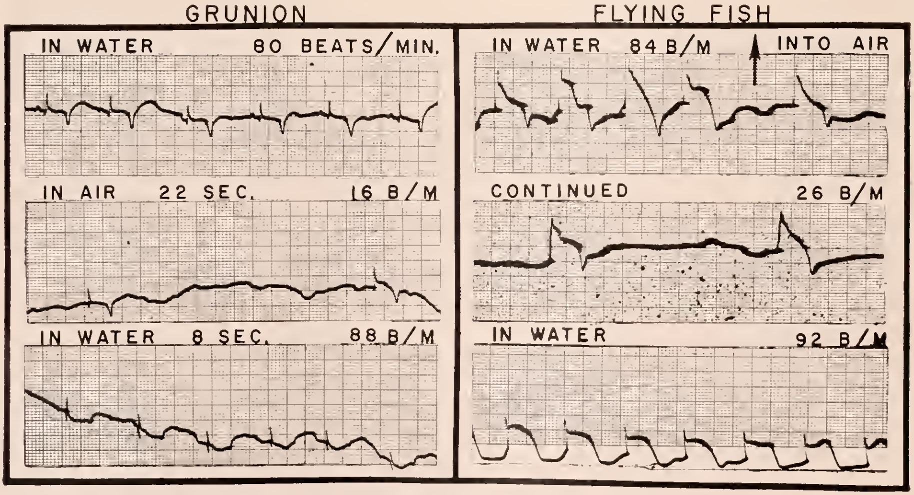

Figure 4. Electrocardiograms of grunion and flying fish in and out of water.

13 seconds, and compound flights of 28 seconds. (A flight of 42 seconds has been reported.)

When flying fish are taken out of water a pronounced bradycardia occurs (Figs. 2, 4). This slowdown to one-third of normal continues until submergence initiates a sharp acceleration of rate. Fish swimming in the ocean with attached electrodes displayed similar effects when pulled from the water and returned.

MUDSKIPPER (*Periophthalmodon australis*)

This mudskipper inhabits the brackish and marine mangrove-fringed mudflats of tropic seas in the Australian region. The mudskippers act more like amphibians than fishes, in that they perch on the mud and mangrove roots and seldom stay in water. When approached by humans, they scurry into a mud burrow or skitter away atop the water.

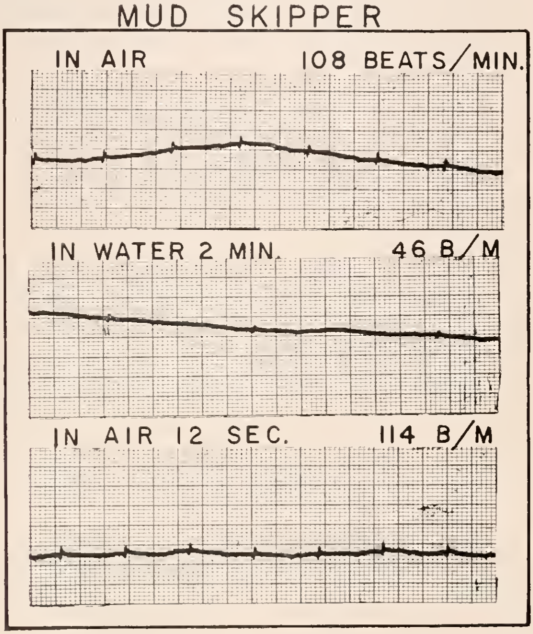

FIGURE 5. Electrocardiogram of a mudskipper in air and in water.

When a mudskipper is put into water it almost always develops a bradycardia, but when it is exposed to air its cardiac rate increases (Fig. 5). It was noted that heart rates decrease after 3–4 minutes in either water or air. Squeezing air from gill pouches, physical agitation, noise, and noxious gas have no noticeable effect upon cardiac rate. It is well known that mangrove mud is highly anaerobic (Scholander *et al.*, 1955). A check on the mud in one burrow showed it to be almost free of oxygen. The anaerobic nature of the burrow is physiologically reflected in a greatly reduced heart rate when the mudskipper is underground (Fig. 6).

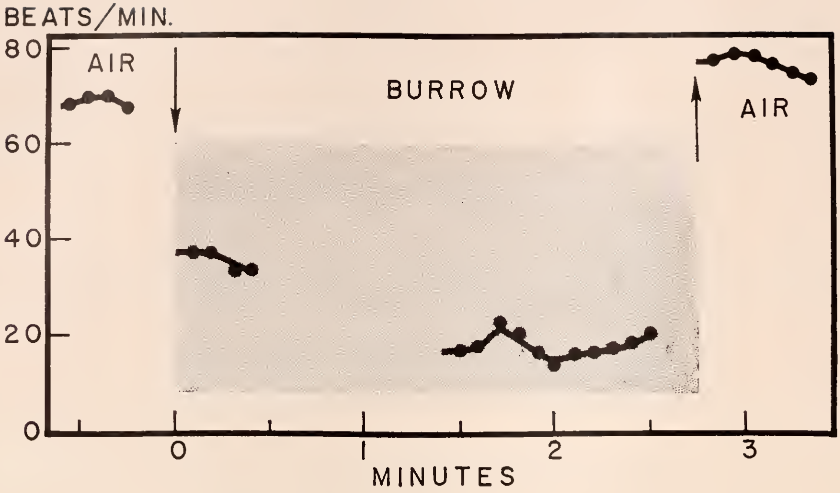

FIGURE 6. Heart rates of a mudskipper in air and in mud burrow.

HAGFISH (*Eptatretus stoutii*)

A hagfish is included in this investigation because it is plausible to assume that it creates for itself an anaerobic condition when it burrows into the bodies of living or dead fish.

Recording of the hagfish's heart rate reveals no bradycardia when it is out of water. Indeed, the rate continued at a steady pace of 44–45 beats per minute in both air and water.

DISCUSSION

The grunion and the flying fish show interesting ability to carry on essential life activities while out of water. The effectiveness of these adjustments is clear. Flying fish escape predators, and mortality of unrestricted grunion during spawning is almost non-existent (Walker, 1952). These fishes respond to aerial situations of low- or high-energy demand with a drop in heart rate. This decrease indicates an overall circulatory reduction, because increased stroke volume cannot offset bradycardia of these magnitudes. Of interest is the similar bradycardiac response exhibited when these fishes enter air and when lung-breathers swim submerged.

Experimental studies suggest that a diving animal's musculature becomes uncirculated and shifts to anaerobic function, thus conserving the oxygen stores (Scholander *et al.*, 1942). It is believed that oxygen-demanding tissues, such as the heart and brain, are selectively supplied with oxygen while peripheral circulation is restricted (Irving, 1939). Pronounced rise of lactic acid in the blood of fishes during the recovery period following air exposure (Black, 1955; Leivestad *et al.*, 1957) supports these views. Our investigation of a few grunion on the beach showed an acute three-fold increase in lactic acid during recovery in water.

Unlike most other teleosts the mudskippers seem physiologically well adjusted to the world of air. They show an evolutionary development to aerial respiration (Carter, 1957; Whitley, 1960), and their bradycardiac response to water is unique among investigated fishes. Indeed, an aquatic existence may now constitute an asphyxic environment for the mudskippers.

The hagfish, an internal predator, presents an exception to the cardiac responses of all investigated vertebrates. Its cardiac rate is unaffected by transitions from water to air. This passiveness, however, reflects a peculiarity in this cyclostome's anatomy—it lacks cardioregulative nerves. The hagfish evidently cannot exhibit cardiac adjustments to the anaerobic conditions it encounters, for in fishes and other animals the heart is controlled by the vagi (Skramlik, 1935; Jensen, 1961).

The sudden changes in cardiac rates of the fishes studied indicate a decisive mechanism of control. An aerial bradycardia is not peculiar to the air-venturing grunion and flying fish, however, for cod (*Gadus morhua*), river trout (*Salmo trutta*), and carp (*Cyprinus carpio*), which are not normally exposed to air, show similar cardiac adjustments (Leivestad *et al.,* 1957; Serfaty and Raynaud, 1958). It has been suggested that the asphyxia causes the onset of bradycardia. However, response of the grunion and the flying fish is so immediate that other factors must be involved. Also, it seems likely that the heart control does not result from simple skin-initiated reflexes, because it is known that psychological factors can induce bradycardia in mammals and in some fishes. Whatever this asphyxial defense mechanism, triggered when animals encounter the unfamiliar world of air or water, likely it aids in survival of the individual and of the species.

This investigation was supported by a research grant, RG-7114, from the U. S. Department of Health, Education and Welfare, Public Health Service; by research grant Nonr-2216(09) from the Office of Naval Research, Department of the Navy; and by research grant G-7476 from the National Science Foundation. The mudskipper was studied in Australian waters on the 1960 Scripps Institution of Oceanography expedition to South China Seas, sponsored on the Public Health Service grant.

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Summary

1. Cardiac frequencies of fishes that normally expose themselves to asphyxic situations were studied.

2. The grunion and the flying fish displayed pronounced bradycardia upon exposure to air with rapidly regained rate when immersed.

3. The air-inhabiting mudskipper responded to water with a heart slowdown and a speedup in air.

4. The hagfish, which lacks cardioregulative nerves, showed no cardiac frequency compensation when placed in air.

5. Comparison is made with the asphyxial adjustments exhibited by land animals when they swim submerged.

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MECHANICS OF THE LIGAMENT IN THE BIVALVE SPISULA SOLIDISSIMA IN RELATION TO MODE OF LIFE

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The bivalve shell is closed by the action of adductor muscles. These, the largest muscles in any bivalve, have no single antagonists but can be stretched by several mechanisms, which include the elasticity of the horny hinge ligament and several kinds of hydraulic systems. The relative importance of each method varies in different types of bivalves.

The elastic ligament connects the shell valves dorsally and is under strain when the valves are closed. The strain is tensile in the outer part of external hinge ligaments, but corresponds to compression in internal ligaments, or resilia, and in the inner layer of others. Functional morphology and development of the ligament have recently received considerable study (Owen, Trueman and Yonge, 1953; Owen, 1953, 1958, 1959a, 1959b; Trueman, 1949, 1950, 1951, 1953, 1954; Yonge, 1953, 1955, 1957), and the detailed investigations of Trueman have included mechanical analyses of the operation of the ligament in certain genera, notably *Ostrea, Pecten* and *Mya* (Trueman, 1951, 1953, 1954). The force derived when the ligament is under strain tends to open the valves, *i.e.,* acts against the adductor muscles, and Trueman (1953, 1954) terms this the "opening moment" of the ligament.

The forces which can be grouped as "hydraulic" means of shell opening are of two kinds, derived respectively from compression of (a) body-fluids in the "haemocoele," and (b) water in the mantle-cavity. The first category includes the protrusion of the foot, used to force the shell valves apart in adult *Cardium edule* and *Anodonta cygnea,* and in the young of many bivalves including *Spisula solidissima,* but could also include the distension of fused ventral edges of the mantle. The second, water pressure, is used particularly in those bivalves where extensive fusion of the mantle margins has occurred (Yonge, 1955, 1957). Hunter (1949) showed that the method of boring in *Hiatella gallicana* depended on the shell valves being forced apart by water pressure inside the mantle cavity. The use of water pressure in the mantle cavity, for extending the siphons by contraction of the adductor muscles, was later investigated in detail in *Mya arenaria* (Chapman and Newell, 1956; see also Chapman, 1958).

Among the larger burrowing bivalves, *Mya arenaria* and *Spisula solidissima* offer a number of contrasts in their modes of life. The former is a sedentary "deep-burrower," almost immobile in adult life, the latter an active burrower, one of the so-called "surf-clams" living just within the substrate. The mechanics of the ligament (Trueman, 1954) and the hydraulics of the mantle cavity (Chapman and Newell, 1956) have been investigated in *Mya*. In spite of other differences, *Spisula* has a ligament structurally similar to that of *Mya*. The data presented in the present paper result from an investigation of the mechanical characteristics of the ligament in *Spisula solidissima* at Woods Hole, and include hysteresis curves and determinations of the opening moment in a range of growth stages (sizes). These data are then discussed in relation to the earlier work of Trueman and others, with special reference to the marked differences between *Spisula* and *Mya*.

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MATERIAL AND METHODS

Specimens of the surf-clam, *Spisula solidissima*, were obtained from a locality on the shores of Martha's Vineyard known to Mr. Milton B. Gray and one of the present authors (D.C.G.). All the material used was collected by hand from depths of 10–13 feet (3–4 m.) by D.C.G., using a face mask and snorkel, and a range of sizes was deliberately selected to include specimens of the largest clams in the population. In this locality, clams occur up to 18 cm. maximum shell length, with a wet weight of 860 g. (with shell, but drained of mantle cavity water). Little or nothing is known of the age of such clams. The bottom deposits are of relatively clean, coarse-grained sand (see below), and the clams are living within the first 12 cm. of the substrate (*i.e.*, with only the posterior edges of the shell and the short tentacle-fringed siphons showing). A typical water temperature by day in summer at the bottom would be 19.5° C. (surface water, 20° C.), and the experimental work on the ligament was done at room temperatures from 22.0 to 24.5° C.

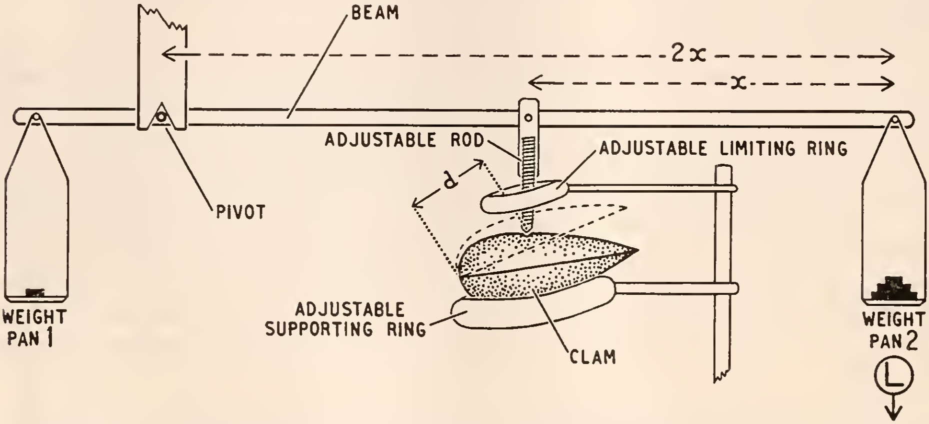

FIGURE 1. The apparatus used to measure the mechanical characteristics of the ligament in *Spisula* (slightly modified from Trueman, 1951, 1953). The pivoted beam is counterbalanced by weights in pan 1, and is so arranged that the force applied through the adjustable rod to the centroid of the upper valve is twice the load (L) in pan 2. The actual moment applied to the ligament also depends on the distance (d) between the pivotal axis of the ligament and the point of application (*i.e.*, the valve centroid). The diagram is not to scale. For further details, see text.

Hysteresis curves and closing and opening moments for each ligament were determined on clams in which all the muscular and other tissues connected to the right shell valves had been cut. This was done with the clam so held that the valves could not gape beyond an angle natural in life. With the left valve firmly supported, loads were applied to the centroid of the right valve by means of a hinged counterbalanced beam (see Figure 1.) This apparatus is essentially similar to that used by Trueman (1951, 1953) for measurements on the ligaments of *Ostrea, Pecten* and other bivalves and is so arranged that the actual load applied to the centroid of the upper (right) valve is twice the load (L) in pan 2. Thus each moment applied to the ligament, M in g. mm., can be derived from the expression $M = d\ (2L + W)$, where d is the distance (in mm.) from the point of application (the valve centroid) to the pivotal axis of the ligament between the shell valves, L is the applied load and W the weight of the upper (right) valve (both in grams). Angles of gape, both in living clams, and during the loading experiments, were determined by measurement of the maximum distance between the ventral edges of the valves with an internal caliper. This distance was used with the shell height, measured from the hinge line to the ventral edge, to derive an angle in radians, later converted to degrees (see Table I). The closed volume of each clam was subsequently determined by displacement weighings, the cleaned valves being filled with plaster of paris.

RESULTS

In most experiments, before loading of the ligament was begun, the valves had been held for some time at an angle of gape about equal to the gape in life of that particular clam. The load applied to the right valve was then increased gradually until the valves were firmly closed, angle of gape and corresponding load being noted at appropriate intervals. The applied load was then gradually decreased until the valves re-opened, and this unloading continued until the natural gape was again approached. Final detection of the points of closing and of opening of the valves was by use of a feeler gauge of thin card at several points around the ventral margin. This loading and unloading sequence was repeated. (In most cases two to three runs per clam sufficed—as the results were extremely consistent, particularly as regards the load at the point of re-opening.) It was found experimentally that the load at opening had not changed several hours after excision of the living tissues. In practice the loading and unloading experiments on each clam were completed within 15–20 minutes. The loads (L) corresponding to each measured gape were then expressed as moments (M) applied to the ligament, using $M = d(2L + W)$. Each gape was converted to an angle in degrees. When the applied moments are plotted against the angles of the loading and unloading sequence for each clam, hysteresis curves result. Figure 2 shows such plots of angle of gape against moment applied for two of the 35 clams of the main experimental series, one (no. 3) very large and one (no. 10) relatively small. Since the ligament is under maximum stress when the valves are completely closed, this "zero gape" corresponds to the maximum force applied. It is therefore convenient to show the closed position (with maximum applied moment) at the right of each graph (as was done by Trueman, 1953, for similar results). Certain features are common to all such curves. Increasing load decreases the angle of gape (section a) until the valves are closed (zero gape—at the right of each figure). The load which just closes the valves must be somewhat decreased before the valves re-open (vertical part between **a** and **b** at the right of the figure). Decrease of load then gives increasing gape (the first unloading curve—**b**), until the "natural gape" is again approached, when the load is increased again (second loading curve—**c**) to shell closure, then again decreased (second unloading curve—**d**). Subsequently the maximum angle of gape (with *no* load) was determined for each clam, and this value is shown connected to each second unloading curve (**d**) in Figure 2. In successive loadings the applied moment for any given angle of gape is reduced,

TABLE I

Natural and maximum angles of shell gape, mean loads at opening, and calculated opening moments for the ligament, in Spisula

Animal No.	Volume, V, ml.	Gape in life, degrees	Maximum gape with *no* load, degrees	Mean load at opening, L, g.	Right valve weight, W, g.	$(2L+W)$, g.	Centroid of shell to centroid of ligament, d, mm.	Opening moment, $M = d(2L+W)$, g. mm.	M/V, g. mm. per ml.
1	218	8.3	18.2	1260	64	2584	46	118,864	545
2	380	8.2	25.0	2240	118	4598	54	248,292	653
3	761	10.5	23.2	2930	191	6051	66	399,366	525
4	105	10.2	25.2	598	27	1223	43	52,589	501
5	290	7.0	24.1	1953	88	3994	48	191,712	661
6	449	9.0	22.1	2963	151	6077	53.5	325,120	724
7	553	8.3	22.3	2188	182	4558	64	291,712	528
8	634	8.4	18.1	3277	213	6767	58	392,486	619
9	160	9.6	22.9	1129	44	2302	37	85,174	532
10	118	8.1	19.3	773	31	1576	37	58,312	494
11	465	8.8	18.1	2630	161	5421	60	325,260	699
12	452	8.1	20.1	2735	135	5605	60	336,300	744
13	336	9.6	24.1	2095	115	4305	51.5	221,708	660
14	445	9.7	19.4	2457	126	5040	55	277,200	623
15	479	9.0	21.9	2195	141	4531	60	271,860	568
16	392	9.2	22.2	2288	116	4692	55	258,060	658
17	549	9.1	20.3	2340	169	4849	62	300,638	548
18	459	9.1	20.0	2887	145	5919	58	343,302	748
19	389	11.0	25.8	2265	123	4653	52	241,956	622
20	404	8.2	20.4	2275	136	4686	59	276,474	684
21	219	7.4	22.3	1255	65	2575	51.5	132,613	606
22	143	7.5	24.3	803	40	1646	41	67,486	472
23	179	9.8	20.7	1207	59	2473	40.7	100,651	562
24	244	8.2	23.0	1703	71	3531	47	165,957	680
25	344	8.3	23.1	1835	103	3773	56	211,288	614
26	296	8.3	25.2	1405	89	2899	50	144,950	490
27	385	9.7	28.7	2563	112	5238	54	282,852	735
28	426	8.6	23.2	2183	124	4490	55	246,950	580
29	200	6.7	22.5	945	50	1940	44	85,360	427
30	237	8.0	20.7	1505	58	3068	48	147,264	621
31	219	8.8	20.4	1355	57	2767	47	130,049	594
32	214	7.7	23.1	1383	60	2826	46	129,996	607
33	358	9.6	21.5	1900	110	3910	53	207,230	579
34	423	7.3	23.6	2335	142	4812	58	279,096	660
35	295	7.2	18.4	2150	100	4400	50	220,000	746

Mean of M/V = 608.83 g. mm. per ml.

i.e., the second loading curve (**c**) lies to the right of the first (**a**). On the other hand, successive unloading curves (**b** and **d**) are closely similar, and the applied moment when the valves first re-open is nearly constant. Trueman (1953) has pointed out that the size of the area enclosed by such hysteresis loops (*i.e.*, between loading and unloading curves) provides a measure of the efficiency of a ligament as regards its elastic properties. The smaller the area, the less "damping" occurs: the more efficient is the ligament. Hysteresis loops for ligaments of *Spisula solidissima* can be compared in this respect with those published by Trueman (1953). The ligament of *Spisula* would appear to be more efficient than those of *Mya arenaria* and *Anodonta cygnea,* but considerably less efficient than those of *Pecten* and *Chlamys.*

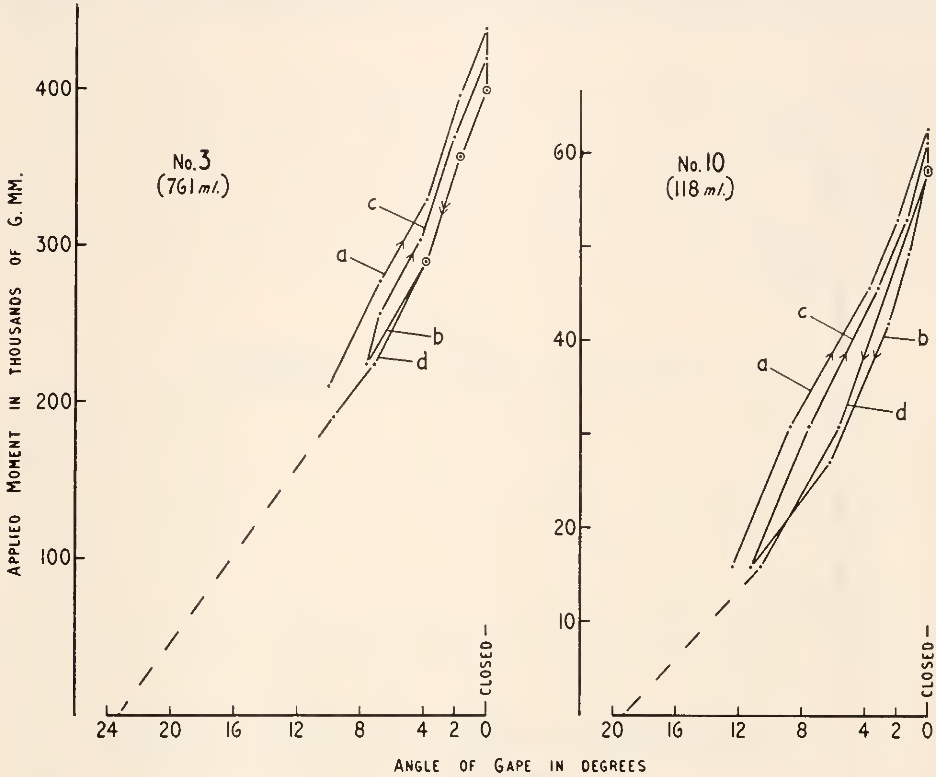

FIGURE 2. Graphs showing the relation between moments applied to the ligament of *Spisula* and the resultant angles of gape of the valves. The shell valves are completely closed at the right of each graph (at maximum applied moment). Each clam was subjected to increasing load (**a**), followed by unloading (**b**), a second loading (**c**) and a second unloading (**d**).

Hysteresis curves are not provided here for all 35 clams of the main series but their mean loads at opening (always consistent values) and their calculated opening moments are shown in Table I. It should be noted that, in these experiments on *Spisula,* each actual load at opening was measured, whereas many of the figures for the opening moments of different bivalves presented by Trueman (1953, 1954) are estimated from closing loads, which alone were measured. In the present work on *Spisula,* at least, it is clear that the load at opening gives a much more consistent value for each ligament than the closing load. Considerable manipulation of any ligament, provided the valves were kept within a "natural" angle of gape, did not appreciably alter the opening load. Even repeated "clapping" of the valves at intervals of about a second produced little change in this measure of elasticity of the ligament. Similar constancy was remarked by Trueman (1953) in experiments on *Chlamys, Pecten,* and *Anodonta.*

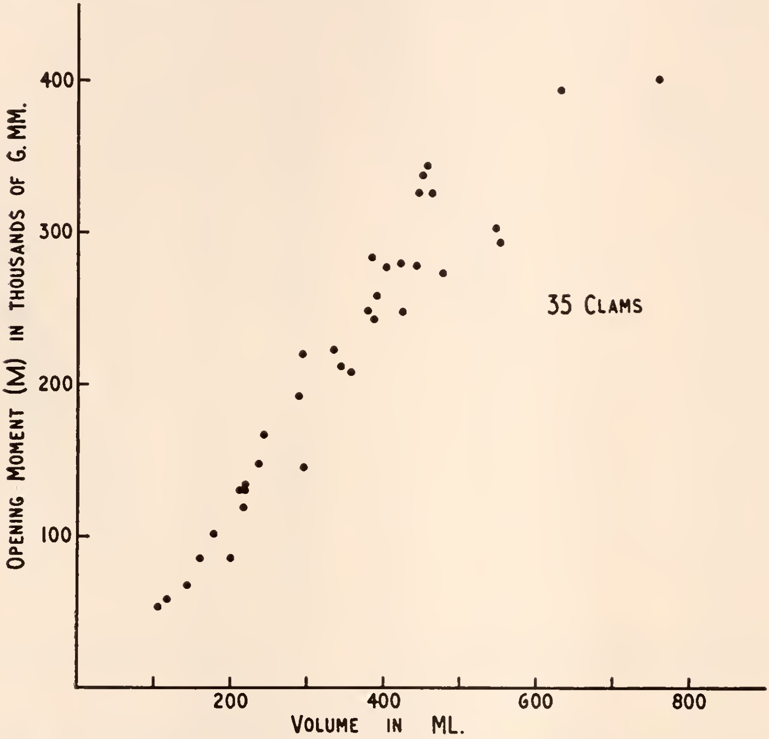

Figure 3. Plot, for *Spisula* of different sizes, of the opening moments exerted by the ligament in relation to the volume of each whole clam. For further explanation, see text.

In Table I the weight of the right shell valve (W) and the distance from the axis of the ligament to the point of application of the load (d) are both shown. From these and the mean load at opening (L), the opening moment (M) is derived using $M = d(2L + W)$. The opening moment of the ligament varies with the size of the clam (see Table I). In some cases, Trueman has related closing and opening moments to the surface area of the valves (Trueman, 1951, 1954), and in others to total shell volume (Trueman, 1953). For the main series of *Spisula* opening moments are plotted against whole volumes in Figure 3. The relationship is satisfactory, and so in the last column of Table I the results are expressed as opening moments per unit volume (*i.e.,* M/V in g. mm. per ml.). The mean value for all the determinations on *Spisula* is 608.83 g. mm. per ml. (range = 427–748, s.d. = 83.02 g. mm. per ml.). This is comparable to values for the most powerful ligaments already measured: those of *Mytilus edulis* and *Cyprina islandica* (660 and 550 g. mm. per ml., Trueman, 1953); and is considerably greater than that for *Mya arenaria* (174, Trueman, 1954) and those for the efficient swimmers, *Pecten maximus* and *Chlamys opercularis* (160 and 142, Trueman, 1953). Thus, in the range of bivalves already studied, the strength of the ligament in *Spisula solidissima* is comparatively great, and its "efficiency" is at an intermediate level. More detailed comparisons between *Spisula* and *Mya arenaria* are made in the discussion below.

Table I also shows, for each clam, the angle of gape of the shell valves measured in life, and the maximum angle of gape (under no load) measured after completion of loading and unloading cycles. For *Spisula* the mean gape in life is 8.6 degrees, and the mean maximum gape 22.1 degrees. This value for the maximum gape is less than that for *Pecten* (approx. 32 degrees) and greater than for *Mytilus* and *Ostrea* (both approximately 10 degrees). As shown for the larger clam in Figure 2, this maximum gape usually corresponds fairly well to an extrapolation of the loading curves, *i.e.*, the applied moment (or, in life, the force exerted by contraction of the adductor muscles) remains more or less constant per degree of gape. In comparing *Spisula* with *Mya* in terms of the mechanical properties of their ligaments, and their very different modes of life, it will be necessary to refer to the types of substrate in which they live. *Spisula solidissima* usually occurs in clean, coarse-grained sand—a surf-shore "shifting-sand." The muddy soil inhabited by *Mya* is usually more consolidated. Mechanical analysis was carried out on samples of sand from the *Spisula* locality, and of soil from a *Mya* bed in the Woods Hole area, and the results appear in Table II. The *Mya* substrate contained some gravel and small stones which accounts for there being 12% of the sample in the > 2 mm. fraction. Apart from this, most of the *Mya* soil lies in the 0.25–0.5 mm. range, while the bulk of the *Spisula* substrate is in the 0.5–1 mm. range. From these samples, *Spisula* seems to live in a significantly coarser and much more uniform substrate. No measurement of the mechanical resistance of these soils was made in the field, but fingers can be moved very readily through the *Spisula* sand, but only with some difficulty in a *Mya* soil.

TABLE II

Particle size in substrates occupied by Spisula solidissima and Mya arenaria: percentage of sample by weight in each size category

Size range of particles in mm.	<.062	.062–.125	.125–.25	.25–.5	.5–1	1–2	>2
Mya soil	.003	.11	11.68	60.96	9.19	5.97	12.07
Spisula soil	.008	.003	.38	17.34	81.6	.59	—

DISCUSSION

As Trueman has pointed out, if the opening moment of the ligament is measured in a range of bivalves, and then assessed in terms of individual size, the range of values cannot be directly related to habitat substrate alone. In a few bivalves, notably attached forms (*e.g., Ostrea,* Trueman, 1951) and actively-swimming forms (*e.g., Pecten,* Trueman, 1953), the ligament is clearly the principal mechanism used to open the valves. As noted above, various "hydraulic" means of shell-opening are relatively more important in other bivalves. In *Mya arenaria,* a deep-burrowing bivalve, Trueman (1954) found that the opening moment of the ligament is inadequate to open the valves when the animal is buried in a typical substrate. Chapman and Newell (1956) showed that the extension of the siphons in *Mya arenaria* depends on water pressures generated by contraction of the adductor muscles acting on the enclosed water of the mantle cavity. Conversely, retraction of the siphons, with all mantle openings closed, will result in opening of the shell valves.

Turning to consideration of *Spisula solidissima,* the present work shows that its ligament is much stronger than that of comparable *Mya arenaria.* The mean opening moment for *Spisula solidissima* is 609 g. mm. per ml., while that for *Mya* is 174 g. mm. per ml. (Trueman, 1954). The extent of "damping" in a complete loading and unloading cycle is also less in the ligament of *Spisula,* the elasticity of which is thus more "efficient" in this respect. In contrast, it is unlikely that hydraulic forces are as important in shell-opening in *Spisula* as in *Mya.* In *Spisula solidissima* the ventral edges of the mantle margins are structurally free (*i.e.,* unfused) between the pedal opening and the base of the siphons. As noted by Yonge (1948) and Kellogg (1915) functional (as distinct from structural) enclosure of the mantle cavity in *Spisula* is produced by close apposition of the free mantle edges posterior to the pedal opening. Although this functional ventral closure is undoubtedly effective in relation to the feeding and cleansing mechanisms of the clams, it is unlikely to be as efficient as the muscular fused ventral surface of the mantle of *Mya* in allowing use of a "hydraulic skeleton." Further, in those bivalves in which increase of water pressure in the mantle cavity can be used to open the valves, the additional capacity for water provided by proportionately large muscular siphons is often clearly important. In an investigation of the boring mechanism in *Hiatella,* Hunter (1949) noted that the increased pressure in the mantle cavity, and the consequent forcing apart of the shell valves, was brought about by the closure of all the openings of the mantle, followed by a retraction of the siphons. Thus, in *Spisula solidissima,* with the mantle lobes effectively unfused along their ventral margins, and with relatively small, inextensile siphons, hydraulic forces utilizing the water of the mantle cavity are unlikely to be important in shell-opening. As noted above, another type of hydraulic mechanism which can be involved in opening movements of the valves depends on body fluids within the foot and pallial edges. The importance of changes in the distension of these organs has not been investigated quantitatively in *Spisula solidissima,* but observations suggest that pedal extrusion is relatively less important as a component of the shell-opening forces in specimens in the present size-range (shell-lengths 9.5–18.0 cm.) than in young animals ($<$ ca. 3.0 cm. long) of the same species.

The resistance of typical substrate to the opening of the clams' valves must also be considered. Trueman has already experimented with *Mya* valves in "soils" of different physical characteristics and provides (1954) some measurements of resistance. As noted above, the *Spisula solidissima* were obtained from a clean, coarse-grained sand, very different from the kind of muddy soil inhabited by *Mya* in the Woods Hole area. Trueman's range of soils did not include any with the surf-shore "shifting-sand" characteristics of the present *Spisula* locality. In a qualitative assessment of the case, although the grain size is larger, the *Spisula* substrate is markedly less resistant to any movement than a *Mya* soil. Trueman (1954) also demonstrated that, in any soil, a greater depth of burial caused an increased resistance to shell-opening. Thus the more powerful ligament of *Spisula* works against less resistance than the ligament and hydraulic forces of *Mya*. It is reasonable to deduce, therefore, that in *Spisula solidissima,* the ligament provides the most important of the forces acting against the adductor muscles and opening the valves. Thus, in the sedentary, deep-burrowing *Mya,* hydraulic means are important in shell-opening and the ligament is relatively weak, while in the active surface-burrowing *Spisula,* the ligament is relatively powerful and hydraulic forces less important.

In *Hiatella,* with a closed mantle cavity, Hunter (1949) noted that the two adductor muscles could act as antagonists to each other, *i.e.*, independently either the anterior or the posterior end of the shell could be closed. In mediating this movement of the valves about a dorso-ventral axis, the water enclosed in the mantle cavity is obviously important. Such movements also do occur in *Mya arenaria,* though they were not reported by Chapman and Newell (1956). In the course of the present work it was noted that similar rocking movements (implying alternate contraction of anterior and posterior adductor muscles) could occur to a slight extent in *Spisula solidissima,* even with the siphons open. In this case the ligament itself must mediate the apparent muscle antagonism.

A final matter concerns the inter-relationships of certain burrowing bivalves. In his published survey of various bivalves, Trueman (1953) gives the opening moment of the ligament for only one species of the family Mactridae, *Lutraria lutraria.* This species is a highly modified deep-burrower, with massive fused siphons. Trueman obtained a value for *L. lutraria,* admittedly from 8 specimens only, of 265 g. mm. per ml. This value is high compared to that for *Mya* (174 g. mm. per ml., Trueman, 1954) though not as high as the present mean value for *S. solidissima* (608.8 g. mm. per ml.). The explanation may be phyletic. It is usually agreed that those bivalves more highly modified for deep-burrowing (*e.g., Mya, Panope* and *Lutraria*) and for boring (*e.g., Hiatella, Pholas, Petricola*) are derived from less specialized shallow-burrowing forms. As Yonge (1948, 1951, 1957 and other papers) has elucidated, the structural and functional adaptations associated with deep-burrowing in bivalves are clearly polyphyletic, and their similarities reflect convergence. Yonge (1948) notes that as a result of its descent from the shallow-burrowing Mactridae, possibly from a form resembling certain *Spisula* spp., the mantle-cleansing mechanisms of *Lutraria* are very unlike those of *Mya,* despite the similarity of external form and habit in the two genera. It can now be postulated that the mechanical properties of the ligament in *Lutraria lutraria* are unlike those of *Mya arenaria,* again as a result of phyletic derivation—in this case from a form with a relatively powerful ligament, like that presently possessed by *Spisula solidissima.*

We wish to record our gratitude to Professor C. M. Yonge, C.B.E., F.R.S., for his kindness in reading the MS of this paper. We are also glad to thank Milton B.

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SUMMARY

1. The mechanical characteristics of the ligament in *Spisula solidissima* have been investigated and, for a range of sizes, hysteresis curves prepared and opening moments calculated.

2. In this species, the opening moment of the ligament can be related satisfactorily to the volume of the clam, and a mean value per unit volume of 608.8 g. mm. per ml. was determined.

3. The mean angle of gape in life of the shell valves of *S. solidissima* is 8.6 degrees, and the mean maximum gape (under no load) is 22.1 degrees.

4. Compared with the structurally similar ligament of *Mya arenaria,* that of *S. solidissima* is about 3.5 times more powerful (in terms of opening moments), and also more "efficient" (as measured by the "damping" of its elasticity in hysteresis loops). Clearly the ligament in *Spisula* provides the most important antagonist to the contraction of the adductor muscles, while in *Mya* water pressures generated in the mantle cavity are important.

5. These mechanical differences reflect the modes of life of the two clams: *Spisula* is one of the so-called "surf-clams," actively maintaining itself just within an unstable substrate; *Mya* is a sedentary "deep-burrower," almost immobile in adult life.

6. A phyletic explanation is suggested for conditions in the deep-burrowing mactrid *Lutraria lutraria,* where the ligament is about 1.5 times more powerful than that of *Mya,* despite their similarities in external form and habit. *Lutraria* is probably derived from a mactrid resembling *Spisula solidissima* and having a powerful ligament.

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THE SWIMMING PLANE OF THE CRUSTACTEAN MYSIDIUM GRACILE (DANA)[1]

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An animal creeping on the ground or on any other solid surface can turn freely only around its dorso-ventral axis. Direct contact with the substrate largely maintains and stabilizes its equilibrium. This is not so, in contrast, for an animal swimming freely in water where such mechanical constraint for turning is absent. Hence the organism's rather labile orientation in space must be controlled continuously so that turbulence of the medium and the inevitable asymmetries in locomotor activity will not cause an erratic course.

In a free-swimming mysid, roll and pitch are under the joint control of orientation transverse to gravity and the dorsal light reflex (Delage, 1887; Bethe, 1895; Bauer, 1908; von Buddenbrock, 1914; Foxon, 1940). Yaw or lateral turning deviations can be checked with the help of polarized light (Bainbridge and Waterman, 1957, 1958; Jander and Waterman, 1960; Waterman, 1960), and when the mysids are positively or negatively phototactic with the direction of a light source (Franz, 1911, 1913; Fraenkel, 1931).

Food finding demands a succession of turning movements inconsistent with a completely rigid control of body position. In mysids, as in bottom-living animals, such searching movements consist almost exclusively of turns about the dorso-ventral axis. Usually rotations about the longitudinal axis and the transverse axis appear only as minor deviations (Cannon and Manton, 1927), although turning about the transverse axis is evoked by hydrostatic pressure changes which result in upward or downward swimming (Rice, 1961). This close control of pitch and roll, coupled with at least temporary extensive spontaneous turnings to the right and to the left, maintains the individual mysid's position within an imaginary plane, which will be referred to as the swimming plane (Fig. 1).

Since the mysid's equilibrium, and hence the inclination and stability of the swimming plane, are controlled both by light and gravity, the question arises how such oriented behavior is altered with changes of intensity and direction of illumination. Of particular interest are those cases in which the two modes of orientation are opposed to each other because behavior in the conflict situation may make possible some inferences concerning the mechanism involved. The present studies are comparable in approach to previous much more detailed investigations on fish orientation (von Holst, 1935, 1950; Braemer, 1957, 1958).

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METHODS

All experiments were performed at the Bermuda Biological Station on *Mysidium gracile* (Dana) collected from a marine pond. A small all-glass aquarium (4 × 8 × 9 cm.), containing some 20 recently caught animals, was illuminated from a distance of 20 cm. by a 6-volt microscope lamp. This produced a circular bright spot with a luminance of 170 candelas/cm.^{2} and subtending a visual angle of 8°. No effort was made to measure the small amount of diffuse light present in the experimental dark room.

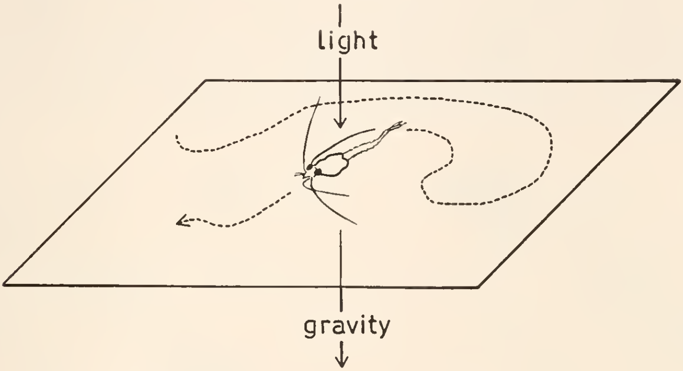

FIGURE 1. The normal swimming pattern of *Mysidium gracile,* showing the swimming plane perpendicular to the direction of light and gravity. Turning occurs almost entirely within this plane.

A series of exposures was taken with a still camera to determine the mysid's angular orientation under each of the different experimental conditions. When the animals were in total darkness, photographs were taken with an electronic flash. The positions of the body axes were measured on the projected negative of the film, summed over 5-degree intervals, and then plotted on polar coordinates as shown in Figures 2–6. During the experiments conditions were changed from exposure to exposure to minimize the effect of any drift in time.

Abbreviations: m = Mean angular measurement.
s = Standard deviation of the mean.
n = Number of individual readings.
$0°$ = Upward direction relative to gravity.

RESULTS

Three experiments were done, one with vertical illumination and two with horizontal.

TABLE I

Data of Experiment 1

Condition	Axes					
	Longitudinal			Transverse		
	m	*s*	*n*	*m*	*s*	*n*
a (dark)	91° ± 1.8°	±24.3°	218	88.8° ± 2.2°	±9.2°	17
b (light)	90.1° ± 1.6°	±27.1°	215	89.2° ± 1.8°	±9.3°	26

Experiment 1. Samples of the mysids' angular orientation were recorded under two conditions: (*a*) in total darkness and (*b*) with a vertical light beam from above. In *a*, only gravity orientation could be effective, whereas in *b*, light could give additional directional clues. The data thus obtained show that under both Conditions *a* and *b* the swimming plane was horizontal. With or without the light from above, the longitudinal and the transverse axes were maintained

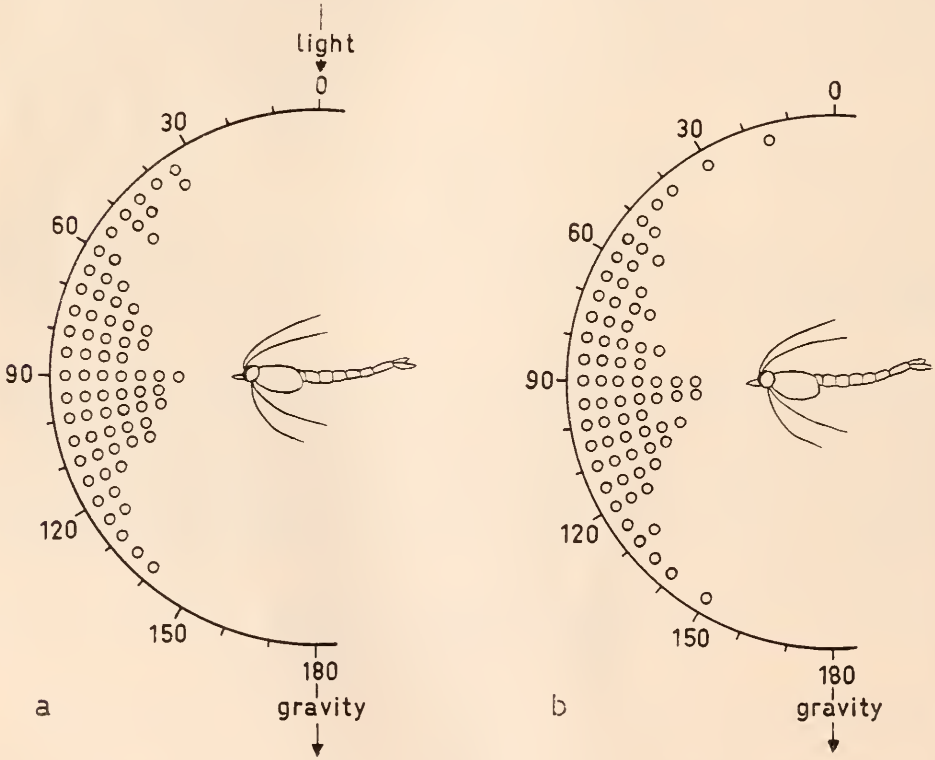

FIGURE 2. Positions observed for the longitudinal axis of swimming *Mysidium* when stimulated by light and gravity (*a*), and when stimulated by gravity alone (*b*). Each small circle represents three readings. Experiment 1.

in the same horizontal positions and, remarkably enough, addition of light as a second source of directional information had no effect on the random fluctuations in the positions of the two axes (Table I and Figs. 2 and 3).

If the accuracy of orientation about the two axes is compared by averaging the data for Conditions *a* and *b*, the fluctuations for the longitudinal axis ($s = \pm 25.1°$) are seen to be 2.7 times larger ($p < 0.1\%$) than those for the transverse axis ($s = \pm 9.2°$). This difference is due to apparently spontaneous up and down undulations of the mysid's swimming path which may be connected with food finding, since such oscillations increase conspicuously after edible particles have been added to the water. Somewhat comparable undulations in the swimming path of *Palaemon northropi* have been observed in the field (Bainbridge and Waterman, 1957).

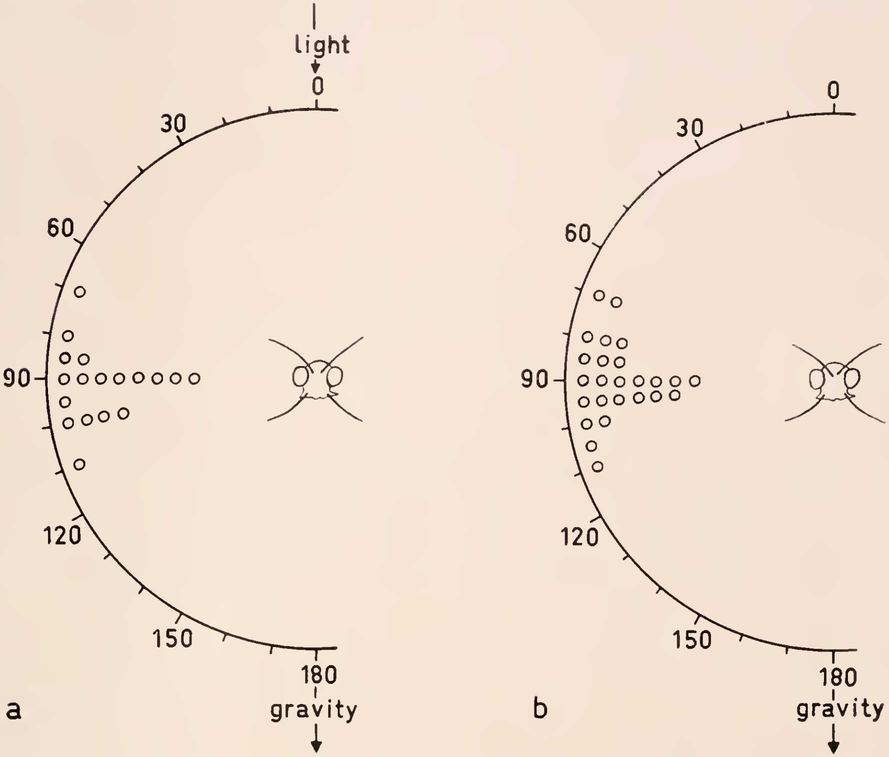

FIGURE 3. Positions observed for the transverse axis of swimming *Mysidium* when stimulated by light and gravity (*a*), and when stimulated by gravity alone (*b*). Each small circle represents one reading. Experiment 1.

This experiment with vertical illumination seems to show that directional light has no effect whatever on the orientation of *Mysidium*. That this is not so can be shown by changing the direction of the incident light as in the following experiment.

Experiment 2. This time the light beam entered the vessel horizontally. The camera was photographing horizontally at 90° to the beam of light. Again records were taken under two conditions. In Condition *a* the light source had its full intensity and in Condition *b* this was reduced by a neutral filter to 6.3% of the original value.

The results are shown in Table II and Figures 4a, b and 5a, b. Under Condition *a*, the longitudinal axis was tipped from its original horizontal position (Experiment 1) approximately 63° upward or downward so that the mysid's dorsal surface was turned partly toward the light source. With the lower light intensity the longitudinal body axis was tilted in the same way but only through about 51° instead of 63°. This reduction of the inclination with reduced light intensity is significant ($p < 1\%$).

The horizontal illumination caused the transverse axis also to be turned away from its horizontal position though to a lesser degree than the longitudinal axis (Fig. 5a, b). Again the direction of tilt was such that the dorsal surface turned partly toward the light source. The degree of this turning was intensity-dependent (55.0° for Condition *a* and 45.0° for Condition *b*) as in the case of the longitudinal axis. Note that again *s* for the longitudinal axis was appreciably greater (± 20.6°) than for the transverse axis (± 13.3°). Turning the light source through 90° from Experiment 1 to Experiment 2 gave rise to turning of the two body axes in the same direction (*i.e.*, dorsum towards the light) but through less than 90°.

TABLE II

Data of Experiment 2

Condition	Axes					
	Longitudinal			Transverse		
	m	*s*	*n*	*m*	*s*	*n*
a (bright)	26.1° ± 1.4° 152.7° ± 3.0°	±20.6°	231 39	35.0° ± 1.6°	±12.4°	57
b (dim)	38.2° ± 1.8° 136.6° ± 3.3°	±20.6°	126 49	45.0° ± 2.1°	±14.2°	47

These facts can only mean that (1) the light beam is indeed being used as a means of orientation, and (2) an additional mode of orientation is present which can use only gravity for a reference direction. This demonstrates again what has been known already (references cited in the introduction), namely that in mysids phototactic and geotactic orientation take place simultaneously. Experiment 1 shows that there is no conflict between geotaxis and phototaxis with a light source in the zenith. From Experiment 2, however, opposed effects of geotaxis and phototaxis can be inferred; this is resolved by the mysids with a compromise reaction. Even in this conflict situation a swimming plane is maintained. Although slanted this plane is still flat and not warped as one might expect in view of the different inclinations of the two body axes involved. This flatness is maintained because progression usually is head first and not oblique or sideways.

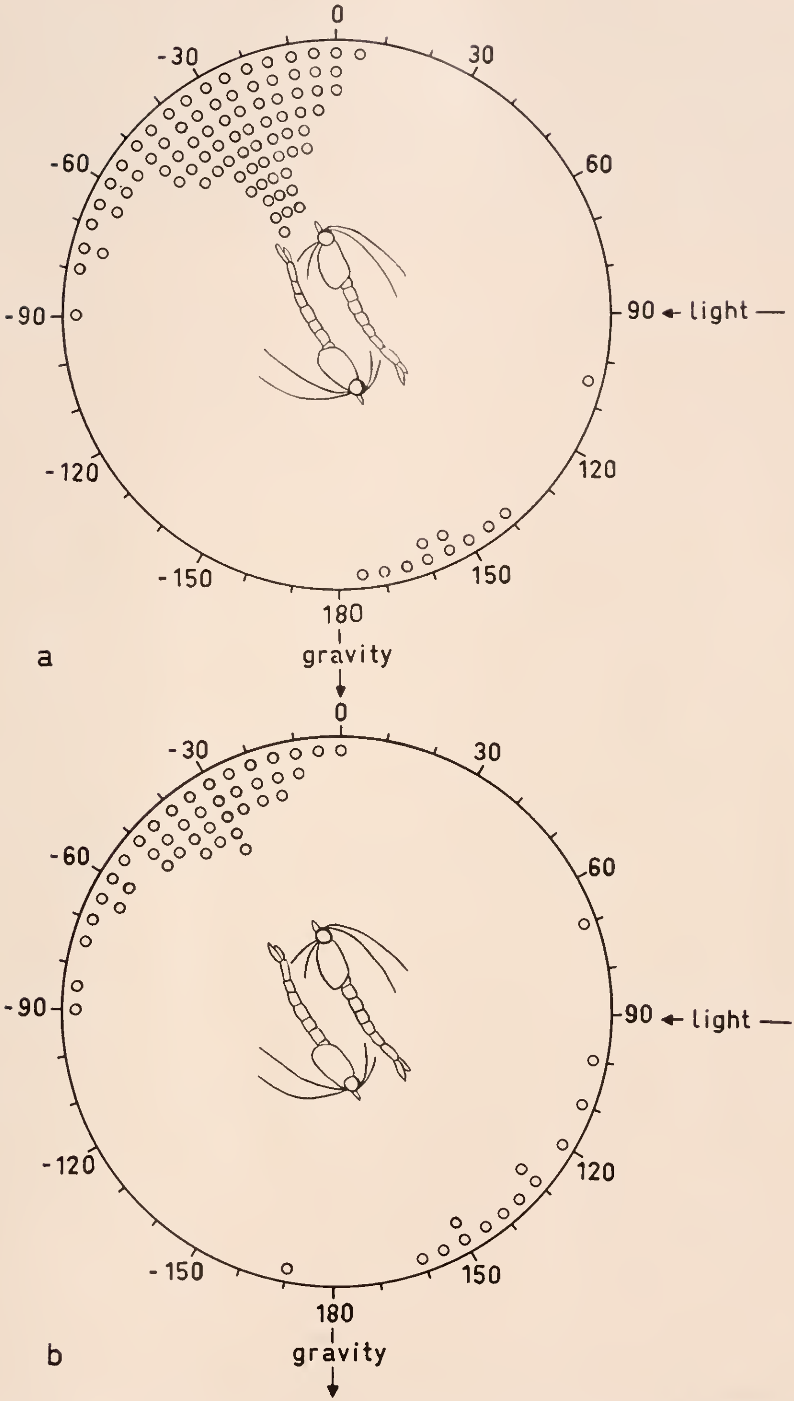

FIGURE 4. Positions observed for the longitudinal axis of swimming *Mysidium* when the light beam stimulates with full intensity (*a*), or with reduced (6.3%) intensity (*b*), horizontally in the plane of the paper. Each small circle represents three readings. Experiment 2.

Experiment 3. Since the dorso-ventral axis in Experiment 2 was always slanted in the field of gravity, geotactic orientation about this axis was possible for geometrical reasons. To determine whether orientation of this sort does in fact take place, the stimulus situation was kept the same as in the previous experiment but the camera was aimed horizontally straight towards the beam of light and hence obliquely faced the ventral side of the swimming plane. The data show (Fig. 6) that upward orientation is predominant although all other directions are possible. This asymmetry occurs because the animals spent much time hovering in a head-up position. The continuous distribution of angular positions found in this experiment contrasts markedly with Experiments 1 and 2 (Figs. 2–5) with their limited angular ranges of swimming directions.

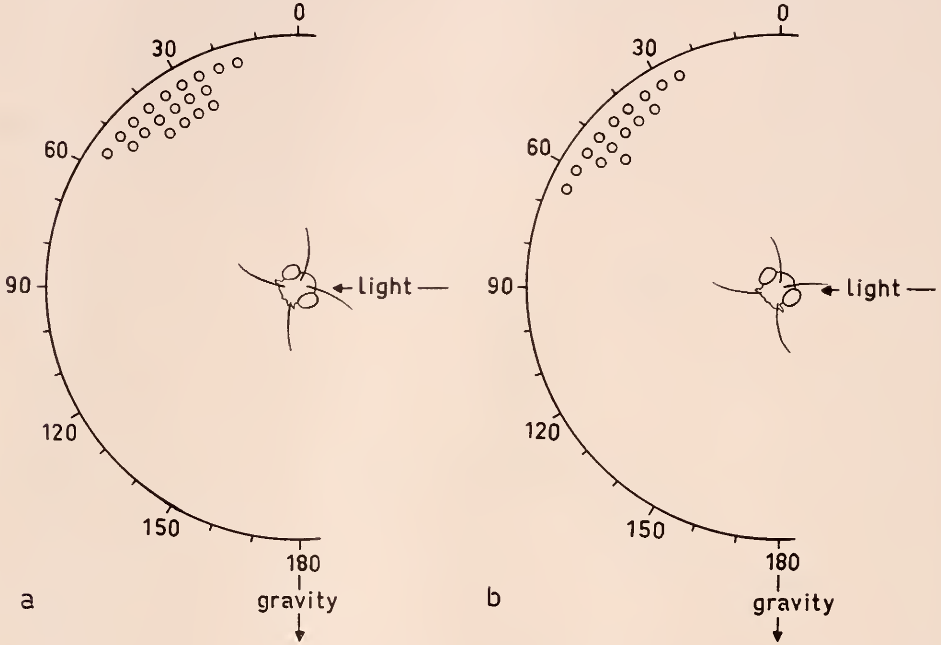

FIGURE 5. Positions observed for the transverse axis of swimming *Mysidium* when the light beam stimulates with full intensity (*a*), or reduced (6.3%) intensity (*b*), horizontally in the plane of the paper. Each point represents three readings. Experiment 2.

The tendency to turn left and right within the slanted swimming plane is therefore greater than the one for turning out of the plane, in spite of the fact that the force of gravity does have a component affecting orientation in such an oblique plane (compare Figs. 4, 5 and 6).

DISCUSSION

Interactions of conflicting turning tendencies in response to simultaneous gravitational and optical stimulation have frequently been studied, *e.g.* in molluscs

(Crozier and Wolf, 1929); arthropods (Yagi, 1928; Schöne, 1954, 1959; Jander, 1957, 1960); and vertebrates (von Holst, 1935, 1950; Braemer, 1957, 1958). Usually all these animals, including the present case of the mysids, integrate centrally the conflicting turning tendencies in a way that determines the particular compromise between the two opposing rotations. Thus the actual position assumed indicates the relative strengths of the turning tendencies induced by the different external stimuli. Evidence of these relations can be obtained from the data of Experiment 2.

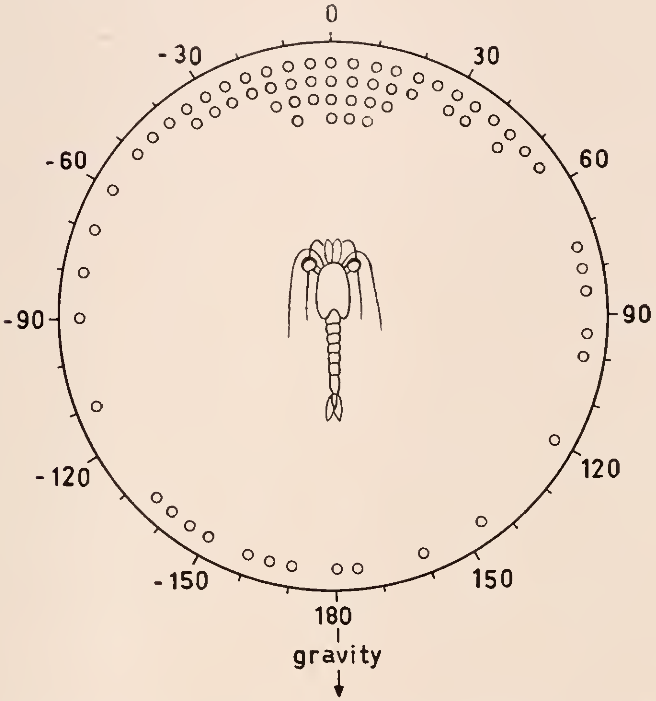

FIGURE 6. Positions observed for the longitudinal axis of swimming *Mysidium* when stimulated by a horizontal light beam (perpendicular to the plane of the paper) and gravity. Each point represents three readings. The inset shows the animal in its most frequent orientation with the longitudinal axis in the plane defined by the directions of light and gravity (the same position is shown from the side in the upper insets in Figure 4). When the longitudinal axis falls outside of this plane, the interaction of light and gravity causes an asymmetry of the eyes in relation to the direction of light as shown in the rare case of a horizontal position of the longitudinal axis illustrated in Figure 5. Experiment 3.

In both Conditions *a* and *b* of Experiment 2, light has more effect on the positions of the longitudinal axis than has gravity. This axis always turned more than 45° in response to shifting the stimulating light beam 90° from the vertical to the horizontal direction (Fig. 4a, b). Using the general rule (Schöne, 1959) that the strength of an animal's dorsal light reflex is proportional to the sine of the angular difference between its actual dorsal direction and the direction of the stimulating light, the relative effectiveness of the two optical stimuli in

Experiment 2 can be estimated. Under Condition *a* (Fig. 4a) the ratio of the gravity-induced turning tendencies to the light-induced ones is equal to the ratio sin 27° : sin 63°, or 1 : 4.05. A similar calculation for Condition *b* yields a ratio of 1 : 1.24. Since the gravitational stimulus was constant the 17-fold increase in light intensity must have been responsible for the 3.3-fold (4.05 : 1.24) increase in the phototactic turning tendency.

A stronger turning tendency with increasing light intensity seems to be the rule for all dorsal light reactions. Such a relationship has been found now in *Mysidium*, in decapods (Schöne, 1954, 1959), in insects (dytiscid larvae, Schöne, 1951), and in fishes (von Holst, 1935; Braemer, 1957, 1958). In contrast, the turning tendencies in positive and negative phototaxes are independent of the stimulus strength in ants (Jander, 1957), bees (Boch, 1957, unpublished), caddis flies (Trichoptera, Jander, 1960) and, on the basis of preliminary studies, in most other holometabolous insects.

Measuring the physiological resolution of conflicting turning tendencies is not the only way of estimating their relative strength. Another method can be derived as follows. The variations or undulations in the position of the body axes measured as standard deviations are the result of two groups of counteracting forces. These are: (1) separate or integrated turning tendencies induced by optical and gravitational stimulation (= tactic forces) which tend to maintain one steady position of the body axes, and (2) the disturbing internal and external randomizing forces, which tend to turn the body axes away from the steady position just mentioned into any position in space. An increase in the strength of the randomizing forces can be expected to augment the standard deviation, and any increase in the strength of the tactic forces can be expected to reduce it.

This second method of estimating the strength of the tactic forces or tendencies with the help of their interaction with randomizing forces has already been successfully applied in caddis flies (Jander, 1960) and *Daphnia* (Waterman and Jander, unpublished). In both cases the results obtained with this method and the other method described above were consistent with each other. However, the mysid results do not yield such a consistent picture.

Experiment 2 demonstrated that both light and gravity together maintain a steady body position. Yet the standard deviations are practically the same in Condition *a* and *b*, and the same holds for Experiment 1. Such stability of the standard deviation must result from a stable balance between the tactic and the randomizing forces. However, Experiment 2 shows that geotactic and phototactic turning tendencies act jointly and that the strength of the latter depends on the light intensity. Hence with the transition from darkness to light in Experiment 1 the combined tactic turning tendencies should be approximately five times larger than the geotactic ones alone (the phototactic turning tendency is four times the strength of the geotactic one according to Experiment 2*a*). But this expected five-fold increase in tactic tendency would reduce the standard deviation of the axial positions five times. This is definitely not true. Similar considerations hold for Experiment 2.

Such an analysis indicates that the tactic turning tendencies controlling position cannot act merely by algebraic addition in opposing the randomizing forces. Most likely one must assume that they are not only added but also divided by their combined strength in a way that can be symbolized by

$$\frac{ax + by}{x + y}$$

when x and y stand for those parts of the tactic forces which depend on the stimulus strength and a and b for those that depend on the stimulus direction. If reciprocal inhibitory processes were at work such an expression might be expected to hold. It should be mentioned in this connection that Schöne (1959, p. 189) was able to show that increasing gravitational stimulation decreases (inhibits) the turning effect of a given light stimulus in crustacean eyestalk movements (*Palaemonetes*, Decapoda). Note also that maintaining an optimum strength for a turning tendency is advantageous, because too strong a response will result in oscillation due to overshooting, and too weak a response will render the mechanism unnecessarily inefficient.

There are several similarities between the equilibrium orientation of fishes (as analyzed by von Holst, 1935) and of the mysids. Both groups use eyes and statoliths in maintaining a swimming plane and both assume compromise positions in the case of conflict. This compromise orientation depends in fishes and mysids, as well as in other Crustacea (Schöne, 1959, 1961), on the intensity of the stimulating light. In both fishes and mysids the relative orienting efficiency of light compared with gravity is greater around the transverse axis than around the longitudinal axis.

The ecological and adaptive significance of having a swimming plane with the properties described may be seen in the following hypothesis. Swimming in a plane could keep the mysids in a layer rich in planktonic food and may help keep swarms together. In the natural habitat one frequently sees mysids in swarms.[3] Also, in nature *Mysidium gracile* has been repeatedly observed in Walsingham Pond, Bermuda, swimming in slanted planes near underwater cliffs. The reason for the tilted swimming plane in this case was undoubtedly the same as in the laboratory experiments. In this way the mysids can graze within a plane near a cliff with reduced chances of colliding with it.

SUMMARY

1. *Mysidium gracile* (Dana) tends to move within a plane, the swimming plane, which is oriented as nearly as possible perpendicular to the directions of light and gravity.

2. Horizontal illumination induces an orientation conflict which is resolved by maintaining the swimming plane in a slanted position intermediate between those positions dictated by the two stimuli.

3. The turning tendencies of the dorsal light reaction increase with increasing light intensities.

4. There is evidence that gravity- and light-induced turning tendencies inhibit each other.

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[3] Other factors related to swarming behavior in a second species of *Mysidium* have recently been reported (Steven, 1961).

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THE EXTENSION OF FERTILIZABILITY AND A HYPOTHESIS ON SPERM ENTRANCE IN SAND DOLLAR EGGS[1]

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The eggs of *Dendraster excentricus* remain fertile for many weeks in the ovaries of females when the mature forms are maintained in running sea water in aquaria in the laboratory. When the eggs are shed or removed from the ovaries to fresh sea water, the fertility usually is lost within 24 hours at room temperature. This early loss in capacity for fertilization (followed by death) is of interest. It does not seem to be because of nutritional deficiencies since the egg carries sufficient materials to insure development well into the pluteus stage.

It has recently been found that cobaltous chloride in sea water will preserve fertilizability in sand dollar eggs for several days beyond the fertile period of control eggs in sea water. Also, it has been found that glutathione, while preventing the action of cobalt in low concentrations, unites with the cobalt in stronger solutions to produce a more effective agent in prolonging the fertile life of *Dendraster* eggs than does cobalt alone (Rulon, 1961a).

The present work deals further with the fertility-preserving action of cobaltous chloride alone and in combination with other sulfhydryl compounds. It also suggests a mechanism in the penetration of the egg in normal fertilization.

MATERIALS AND METHODS

The mature sand dollars (*Dendraster excentricus*) were dredged from Monterey Bay during the months of July and August and maintained in running water in the aquarium room of the Hopkins Marine Station. The eggs were taken by cutting away the oral surface of the animal and allowing the ripe ovaries, exposed to air, to exude their products. The ova were drawn into medicine droppers and transferred to fresh sea water. After testing for fertility (samples exposed to sperm suspensions) the unfertilized eggs were transferred to the test solutions (made up with sea water) in covered fingerbowls. The eggs were kept in the solutions out of direct sunlight and at laboratory temperatures (18 ± 1° C.) for the duration of the experiments. At stated intervals of time (24, 48, etc. hours) samples of eggs were removed from the test solutions, washed in sea water and placed in Syracuse watch dishes where fresh dilute sperm suspension was added. Notes were made on egg jelly, fertilization membranes, cleavage patterns, etc. It previously had been found (Rulon, 1961a) that extended treatment with cobalt in high concentrations

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[1] This investigation was supported by the Graduate School of Northwestern University. The writer is also much indebted to Dr. L. R. Blinks, Director of The Hopkins Marine Station, Pacific Grove, California, for many considerations.

resulted in tight fertilization membranes and aberrant cleavage patterns. Therefore, the criterion of fertilization selected was that of nuclear division following insemination. In some cases this was the only criterion available since following certain severe treatments the fertilization membrane remained unelevated and there was no cytoplasmic division, yet the nuclei divided many times.

During the course of this work it became increasingly evident that the eggs in the experimental solutions gradually aged. With the most favorable concentrations (see Tables I and II) the jelly surrounding the eggs remained intact for the first 8–12 days and on return to sea water elevated fertilization membranes and normal cleavages appeared following the addition of sperm suspension. In time, however, the fertilization membranes ceased to become fully elevated and in many cases were not elevated at all. With tight membranes there was an inhibition of cytoplasmic division although nuclear division could be seen clearly (see Figs. 11–15, Rulon, 1961a). Toward the end of the experiments no fertilization membranes could be detected and in most cases the blastomeres of the cleaving eggs were almost spherical in shape and loosely held together (lack of cohesiveness). Most of the eggs that had been fertilized late in the experiments developed into normal plutei. In some cases, however, the embryos were stunted and granular.

No cytological studies of sperm entrance have been made at this time. It does not seem likely that the return of the eggs from the experimental solutions to sea water is the activating factor since these eggs begin development only after exposure to sperm suspensions. There can be no doubt of the preserving action of the substances used in these experiments since the control eggs in sea water were usually cytolyzed completely by the end of the second day.

EXPERIMENTAL

1. *The effect of cobalt and thioglycolic acid on fertility in sand dollar eggs.* Unfertilized eggs, testing 95% fertile on removal from the ovaries, were placed in a range of concentrations of cobaltous chloride, thioglycolic acid, and a combination of the two. After preliminary experiments in determining effective ranges, the most suitable concentrations were selected and the eggs tested for fertilizability over a period of 25 days.

In Table I are recorded the effects of the agents. The table is condensed since little would be gained by showing the daily percentages of fertile ova. It will be noted that cobaltous chloride ($M/400$–$M/800$) prolongs fertilizability in 55–60% of the eggs for over four days while none of the eggs in the sea water control of thioglycolic acid was fertile at this time (fertility lost in both solutions by two days).

In the combination solutions the fertilizability was enhanced and extended tremendously. The best combination (50 cc. $M/200$ cobaltous chloride plus 50 cc. 0.01% thioglycolic acid) preserved fertility in 50% of the eggs for over 25 days.

2. *The effect of cobalt and cysteine on fertility in sand dollar eggs.* Eggs similar to those tested with cobaltous-thioglycolic acid were placed in ranges of cobalt, cysteine, and cobaltous-cysteine. Again it was noted (Table II) that the controls in sea water and the eggs in the sulfhydryl solutions could not be fertilized at two days while those in cobaltous chloride ($M/400$–$M/800$) showed increased fertilizability (5–50% at 8 days). In six solutions of cobaltous-cysteine the fer-

TABLE I

The effects of cobaltous chloride and thioglycolic acid for various intervals of time on fertilization of the eggs of Dendraster excentricus
(Values given in percentage)

Solution used	4 days	11 days	15 days	19 days	25 days
Sea water (control)	—	—	—	—	—
$M/400$ cobaltous chloride	60	—	—	—	—
$M/800$ cobaltous chloride	55	—	—	—	—
0.005% thioglycolic acid	—	—	—	—	—
0.0025% thioglycolic acid	—	—	—	—	—
50 cc. $M/200$ cobaltous chloride + 50 cc. 0.01% thioglycolic acid	90	80	60	50	50
50 cc. $M/200$ cobaltous chloride + 50 cc. 0.005% thioglycolic acid	80	60	50	—	—
50 cc. $M/400$ cobaltous chloride + 50 cc. 0.01% thioglycolic acid	85	20	—	—	—
50 cc. $M/400$ cobaltous chloride + 50 cc. 0.005% thioglycolic acid	75	1	—	—	—

tility was enhanced, extended, or both. The best combination (50 cc. $M/200$ cobaltous chloride plus 50 cc. 0.125% cysteine) preserved fertility in 50% of the eggs for over 21 days. The one combined solution (50 cc. $M/400$ cobaltous chloride plus 50 cc. 0.025% cysteine) that failed to enhance or extend fertility over an equivalent solution of cobalt alone may have done so because of a disproportionately large amount of sulfhydryl to cobalt.

TABLE II

The effects of cobaltous chloride and cysteine hydrochloride for various intervals of time on fertilization of the eggs of Dendraster excentricus
(Values given in percentage)

Solution used	2 days	8 days	12 days	16 days	21 days
Sea water (control)	—	—	—	—	—
$M/400$ cobaltous chloride	95	5	—	—	—
$M/800$ cobaltous chloride	95	50	—	—	—
0.0125% cysteine	—	—	—	—	—
0.00625% cysteine	—	—	—	—	—
0.003125% cysteine	—	—	—	—	—
50 cc. $M/200$ cobaltous chloride + 50 cc. 0.025% cysteine	100	85	5	—	—
50 cc. $M/200$ cobaltous chloride + 50 cc. 0.0125% cysteine	95	90	85	50	50
50 cc. $M/200$ cobaltous chloride + 50 cc. 0.00625% cysteine	95	90	50	50	—
50 cc. $M/400$ cobaltous chloride + 50 cc. 0.025% cysteine	60	2	—	—	—
50 cc. $M/400$ cobaltous chloride + 50 cc. 0.0125% cysteine	95	80	—	—	—
50 cc. $M/400$ cobaltous chloride + 50 cc. 0.00625% cysteine	95	85	—	—	—

Discussion

Much work has been done on aging eggs in echinoderms and in general it has been found that as the unfertilized egg ages, there is an increase in viscosity and permeability (see Goldforb, Landowne and Schechter, 1937, for references). It is not known that such physical changes destroy fertility but they seem to be related to its loss. Moreover, it should be pointed out that permeability to water or ions is far different from permeability to (or penetration by) spermatozoa.

The fertile life of marine eggs has been extended by factors other than low temperature. Low-calcium sea water (Schechter, 1937; Rulon, 1948) has been found to prolong or enhance fertilizability in *Arbacia* eggs. Ethyl alcohol and dextrose will do the same with unfertilized *Urechis* eggs (Whitaker, 1937). Loeb (1912) found that the fertilizable life of the starfish egg could be extended by cyanide or oxygen-lack. He believed that the egg becomes unfertilizable, in the normal course of events, after a damaging amount of aerobic oxidation takes place, and that such agents as potassium cyanide preserve fertility by slowing down this action.

While low-calcium sea water, alcohol and dextrose may preserve fertility by affecting viscosity, permeability, or nutrition (see above references) in the unfertilized egg, it appears that the action of cobalt may be somewhat similar to that of cyanide and oxygen-lack as described by Loeb. In other words, cobalt and cobaltous-sulfhydryl combinations prevent the loss of fertility from damaging aerobic oxidations.

A tentative hypothesis suggested by this and previous work (Rulon, 1961a) is that in normal oxygenated sea water, soluble sulfhydryl compounds of the egg cortex may be expected to unite with each other by oxidation to disulfide. Simple proteins become united into large stable insoluble protein chains in which the union of the individual members is through -S-S- bonding. In recent years several workers have shown such bonding to form stable protein chains (Jensen, 1959). Such a change at the egg surface may well account for the loss of fertility in aging eggs.

It seems that the contact of the surface of the fertile egg by the acrosome filament (see Colwin and Colwin, 1957) causes physico-chemical changes (probably enzymatic) at the point of contact which, in most cases, permits the sperm to be engulfed. Pronounced oxidation of R-SH to R-S-S-R at the egg surface could presumably render the surface unsusceptible to sperm action (action of acrosome filament). Perhaps up to a certain point in the aging process the sperm causes a temporary reversal (-S-S- to -SH) of the reaction and thereby creates a momentary fluidity at the point of contact and entrance. It is known that -SH increases in the egg following fertilization (see Runnström, 1952, for references). The writer is unaware of any studies made of -SH at the point of sperm entrance and, of course, this hypothesis needs much in the way of experimental support.

It seems likely that cobalt unites with thiol groups at the egg surface, preventing the chain reaction, and in so doing preserves fertility. It has been shown by previous work (Rulon, 1961a) that glutathione renders low concentrations of cobalt ($M/3200$–$M/6400$) ineffective. High concentrations of cobalt ($M/400$–$M/800$) in combination with glutathione, cysteine thioglycolic acid have been found to be much more effective than cobalt alone. Perhaps these cobaltous-sulfhydryl combinations attach to the egg surface in much the same way that $Co^{60}Vit.B_{12}$ combines with alpha and beta globulins of serum proteins and the protein of cerebrospinal fluid (Meyer, Bertcher and Mulzac, 1959). Such union at the egg surface may well prevent the normal deterioration of fertility by oxidation.

Recent experiments by the author (Rulon, 1961b) have shown that vitamin B_{12} itself has fertility-preserving action on these eggs but the effect was far less than those of the proper concentrations of cobaltous-sulfhydryl. It is to be hoped that future work will elucidate some of the many problems posed by these studies.

SUMMARY

1. The eggs of *Dendraster excentricus* lose the capacity for fertilization within two days when removed from mature ovaries and allowed to stand in open sea water at room temperature.

2. Cobaltous chloride ($M/400$–$M/800$) in sea water will preserve fertility in 5–50% of the eggs for as much as eight days.

3. Neither thioglycolic acid nor cysteine hydrochloride was found to have preserving action on fertility.

4. Certain combinations of cobaltous chloride with thioglycolic acid or cysteine were found to preserve the fertility of 50% of the eggs for 21–25 days.

5. It is suggested that cobalt and cobaltous-sulfhydryl combinations prevent the decay of fertility by preventing destructive oxidations, and that sperm entrance normally may be facilitated by the reduction of disulfide to sulfhydryl at the egg surface.

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TISSUE AFFINITY IN AMAROECIUM. II. REAGGREGATION OF THREE PARTIAL ZOOIDS INTO FUNCTIONING SIAMESE TWINS[1]

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A zooid of *Amaroecium constellatum*, when macerated into fragments of tissue in the cavity of its tunic, reassembles its parts into a complete, normal zooid in a period of, approximately, five days. The capacity in tunicates that is responsible for such virtuosity of reconstitution from fragments is the fundamental property of these tissues to "recognize," grow toward, and fuse preferentially with their homologous types. It expresses itself at each level of organization: (1) The dissociated masses of each organ reassemble themselves into their respective organs, slightly smaller than the previous size in accord with the loss of some areas in maceration; (2) the reintegrated organs join together to establish their former systemic relationships; (3) the systems arrange themselves, in the recombination, according to previously existing axes of polarity. Reaggregation, therefore, is directed by the specific histogenetic nature of tissues; alignment is determined by an established axial gradient.

The facility and speed with which any detached fragment of *Amaroecium* regenerates the total organization of the zooid would lead one to expect a mass regenerative activity among the fragments following the process of maceration. Production of multiple zooids from each individual would, in this event, be the outcome. The fact that reaggregation rather than regeneration occurs indicates that affinity among tissues of common histological differentiation dominates over the regenerative process in tunicates. The character impressed on tissues, first by morphogenetic movements at gastrulation and, later, by histological differentiation, is persistent to the point of regulating the type of reaggregation in dissociated masses of tissues. In the case of *Amaroecium,* it marks all regions of an individual originating from a fertilized egg.

The present experiment was performed in order to study the capacity for reaggregation among tunicate tissues when the fragments are derived from several individuals of the same species whose contributing members, therefore, do not share a common embryonic morphogenesis and developmental environment.

MATERIALS AND METHODS

Zooids of *Amaroecium constellatum,* 12 to 18 hours old, were selected for the experiment. At this period of development the larval organs have differentiated into their adult condition and axiation. The siphons have not yet opened and the

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digestive tract is, therefore, free of any sand or extraneous material that might interfere with the subsequent activities of tissues and with the study of their behavior. The tissue is young adult tissue in an animal in which chordate organization is represented in its simplest expression.

Three extended zooids were divided by the sharp cleft of a micro-knife into their component branchial and abdominal halves. Two branchial regions and one abdominal region were removed from their tunics and transferred, by pipette, to a Syracuse watch glass in which two-week-old *Amaroecium* had been cultured. The older zooids were evicted from their tunics and discarded.

The three contributing members were inserted into the cavity of each host tunic where they were mildly macerated for the purpose of bringing their injured surfaces into contact with each other. While no attempt was made to arrange the members in a consistent order, they were inserted, in most cases, in two general patterns: in a direct linear order of branchial-abdominal-branchial regions, or in an abdominal-branchial-branchial order. The three halves were arranged, therefore, in tandem fashion with the abdominal member in the middle, or in the form of a Y, with the single abdominal segment forming the tail of the Y.

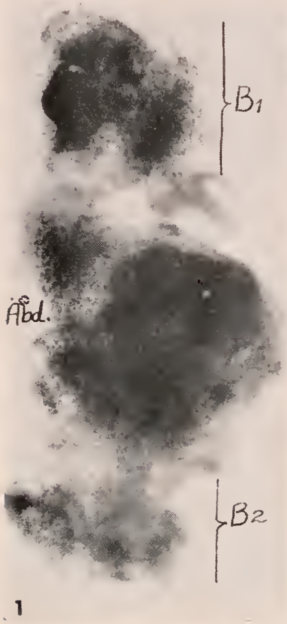
Before the members were combined in the host tunic, the caudal elements of all three contributing zooids were removed, in many cases, completely, in order to provide maximal free space within the tunic for unobstructed movements of the tissue masses. The characteristics that make the tunic of *Amaroecium* an excellent environment for experimental purposes are described in a previous paper (Scott, 1959).

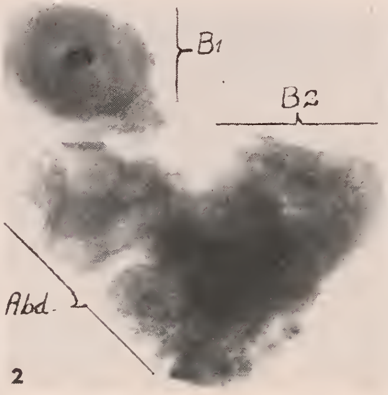
After the three half zooids were macerated in the host tunic, the composites were kept intact by plugging the open tunic cavity with clumps of tunicin from the tests of the contributing zooids (Fig. 3). Numerous such combinations were made in each watch glass and the dishes were then placed in running sea water for optimal conditions of development. They were examined at frequent intervals and the series adopted for critical analysis extended from the time of combination to the reconstitution of Siamese twin organisms capable of feeding; a period covering, at the least, five days from the time of combination to the assumption of functional activity by the organisms. There were 286 such composite zooids assembled, 83 of which, 30%, were used for analytical study. Of the remaining 60%, about 30% regenerated multiple individuals because of the failure of their injured surfaces to come into contact during maceration. The remaining 30% demonstrated fusion of homologous tissues between two of the three component members and separation of the third which regenerated into a complete zooid. About 10% were fatalities. They were infested by scavenger Protozoa or consumed by small crabs in the tank.

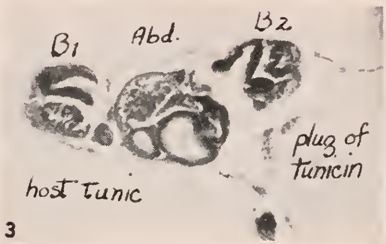
The composite organisms were killed and fixed during this sequence at critical periods of their development. They were fixed in Schaudinn's fluid, heated to 60° C. and poured in generous quantities over the watch glass in order to obtain maximal degree of extension. The organisms for whole mounts were stained with the Feulgen technique. The serial sections were stained in Harris' hematoxylin and counterstained with triosin. All photomicrographs were taken with a Leica "Micro-Ibso" attachment, on 35 mm. Panatomic-X film.

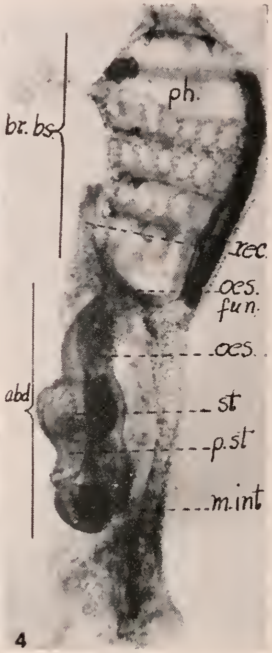
OBSERVATIONS

The aplousobranchiate tunicates possess a systemic organization that adapts them particularly well for dissociation into regions. All structures are clearly visible and distinguishable. In its extended condition the animal can be divided easily into branchial and abdominal halves by the quick cut of a micro-knife. The branchial half of the zooid contains the pharynx with its gill clefts, endostyle and oesophageal funnel with a longer or shorter segment of oesophagus, the contiguous walls of peripharyngeal cavity, both siphons, the neural complex of ganglion and nerves, a piece of the rectal segment of the intestine at the base of the atrial cloaca, and the epidermal mantle. The abdominal half contains the remaining portion of the oesophagus, the stomach, post-stomach, mid-intestine, intestine, epicardium, pericardium and enclosed heart, body cells and enveloping epidermis (Fig. 4).









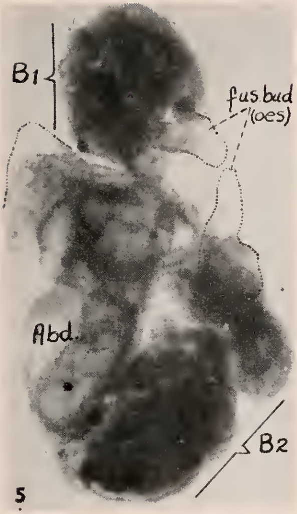
FIGURES 1 and 2. Composite mass of three half zooids at time of combination.

FIGURE 3. Section of composite in the cavity of the host tunic showing the plug of tunic that holds the mass intact.

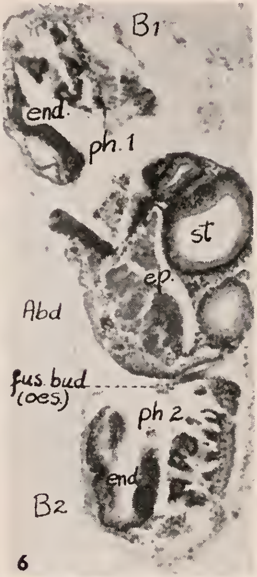
FIGURE 4. The normal zooid at the stage used for the combination. (About 90 ×.)

abd., abdominal fragment; B_1 and B_2, branchial fragments; br. bs., branchial basket; m-int., mid intestine; oes., oesophagus; oes. fun., oesophageal funnel; p-st., post-stomach; ph., pharynx; rec., rectum; st., stomach.

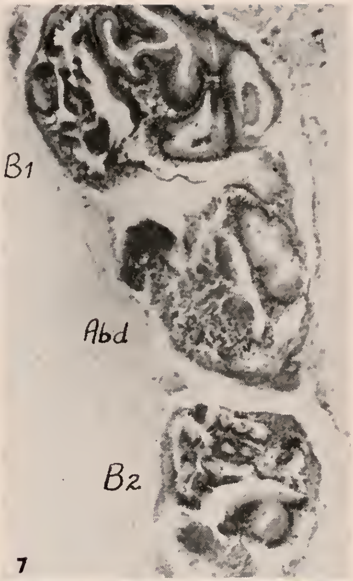
The fact that different lengths of oesophagus may be attached to both halves constitutes the only variable in tissue content in the triple combinations. It introduces no impediment to the process of reconstitution save insofar as it augments the amount of digestive tract and increases the possibility of providing the reaggregate with a second stomach.


B1
fus. bud (oes)
Abd.
B2

5


B1
end.
ph. 1
st
ep.
Abd
fus. bud (oes.)
ph 2
end.
B2

6



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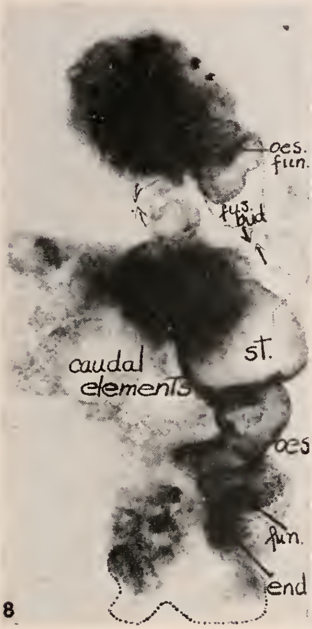
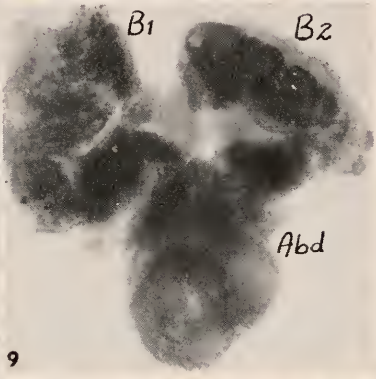

oes. fun.
fus. bud
st.
caudal elements
oes
fun.
end

8



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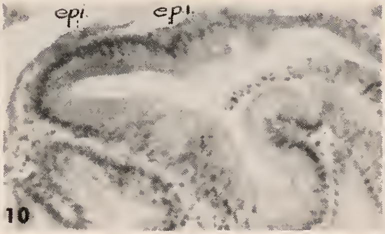

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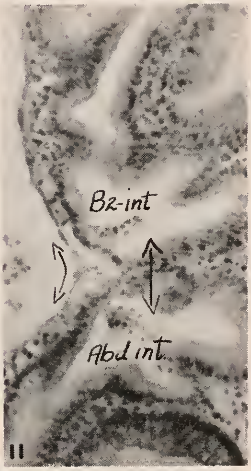
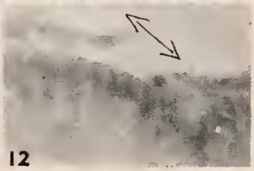

B2-int
Abd int.

11



12

FIGURE 5. Whole mount of composite after six hours.

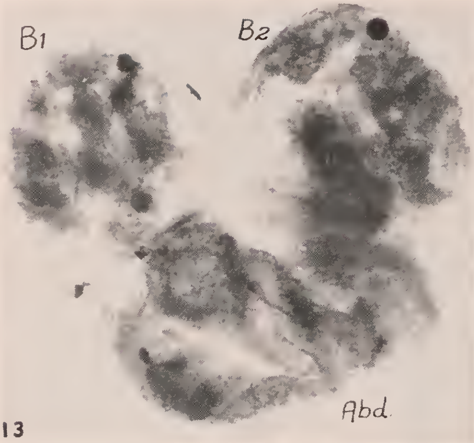
FIGURES 6 and 7. Longitudinal sections, three-hour combinations.

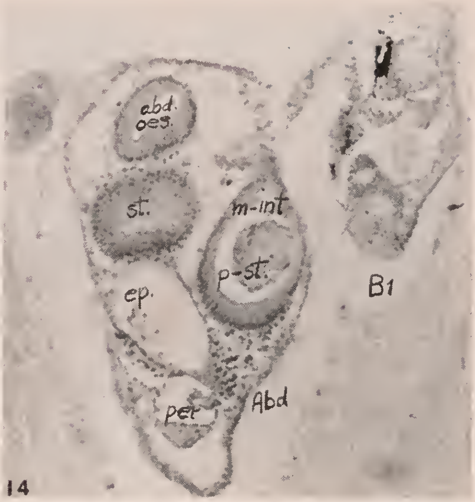
FIGURE 8. Whole mount after nine hours.

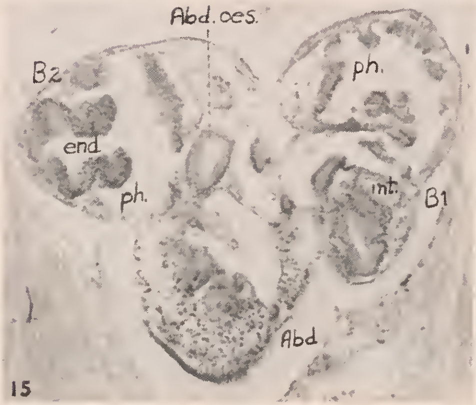
FIGURE 9. Whole mount of three-hour combination.

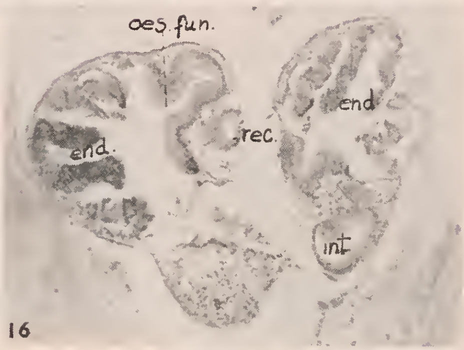
FIGURES 10, 11 and 12. Sections of three-hour combinations showing spreading of epidermis and union of abdominal and branchial intestinal fragments. (About 90 ×.)

abd. int., abdominal intestine; B_2-int., branchial intestine; end., endostyle; ep., epicardium; epi., epidermis; oes. fun., oesophageal funnel; fus. bud, fusion bud of both branchial oesophageal funnels; oes., oesophagus; ph., pharynx; st., stomach.

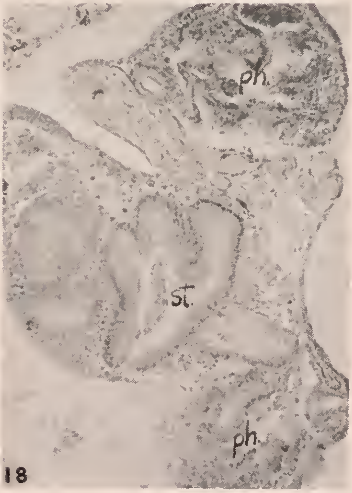

B1
B2
Abd.
13

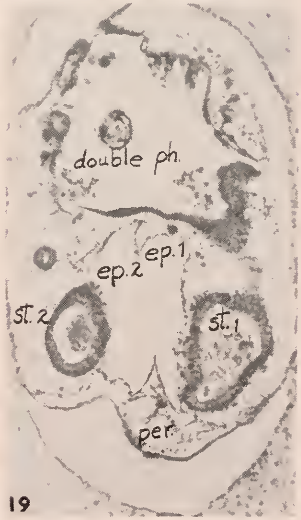

abd. oes.
st.
m-int
p-st.
ep.
B1
per
Abd
14


Abd. oes.
B2
ph.
end
ph.
int.
B1
Abd
15


oes. fun.
end
end.
rec.
int
16

B1
Abd.
B2
17


ph.
st.
ph.
18


double ph.
ep. 1
ep. 2
st. 2
st. 1
per.
19

FIGS. 13–19.

When the three contributing halves are assembled, the composite is a mass of disorganized tunicate tissues (Figs. 1, 2). By examining the living forms, the various organs can be discerned and the degree of disruption ascertained. The cilia of gill clefts and digestive epithelium continue to beat and the heart, if it is not destroyed in mincing the members together, continues to contract during and after the period of assembling and maceration. The smaller fragments of tissue that are killed by the macerating procedure, some of the body cells, and remnants of caudal elements are directed, by ciliary action, into the gaping cavities of digestive tract. The rest of this cellular detritus collects in the cavity of the tunic at the surface of, or between, the dissociated members.

Within the short period of three hours in running sea water, the composite animals show activities of fusing their multiple parts together (Figs. 6, 7, 9). The interrupted ends of epidermis commence immediately their "spreading" movements into the spaces between the fragments. The marginal cells change in shape and develop elongate processes at their free ends, the processes stretching toward similar ones on the free edges of the neighboring patches of epidermis (Figs. 10, 11, 12). This behavior of mantle is typical of *Amaroecium* when it is reaggregating its parts into a whole after dissociation of a single individual. By such means an epidermal envelope is quickly established about the fragments of the zooid (Scott, 1959). The undisturbed mantle cells become more shallow as they spread over wider areas but they retain their optically visible histological characteristics.

The free cells in the cavity of the host tunic collect about the implanted composite and the cells in the tunic proper migrate toward its inner border, congregating there in a pseudo-epithelial layer (Fig. 6). The space between the implant and the tunic remains open during the first day of reaggregation. The space is open for two reasons; first, the animals whose parts are assembled are smaller than the host zooid whose tunic is used, in spite of the fact that three halves of the smaller zooids are used; and second, the assembled members are in a state of contraction. Their tissues slowly relax in the course of the first two days and, eventually, their joined parts fill the cavity. Upon restoration of the mantle epithelium, the implant adds its secretion of tunicin to the host tunicin and thus the mutual adoption of tunic and composite zooid is completed.

The epidermis acts more quickly than the other tissues in the process of reunion. In the early part of the first day, however, segments of digestive tract are recovering from their tight coils and they extend themselves in various directions (Figs. 6, 7, 10, 11). The injured surfaces release clusters of migrating cells that revert, in their general appearance, to a condition of pre-differentiation; that is, they lose their discernible marks of differentiation (Figs. 25, 29). Reconstitution of each organ in the digestive system is accomplished by mutual extension of corresponding

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FIGURE 13. Whole mount of union one day old.

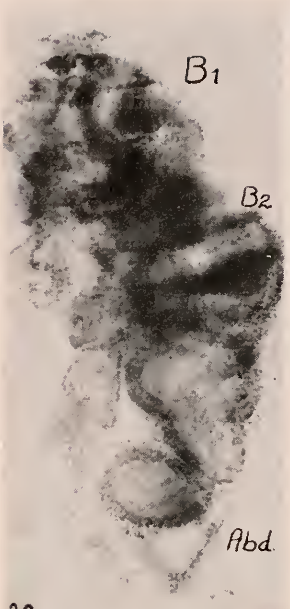
FIGURES 14, 15 and 16. Sections through one-day-old twin.

FIGURE 17. Whole mount of one-day showing linear alignment of member halves.

FIGURE 18. Section through the same type of combination.

FIGURE 19. Section through a four-day twin with fused pharynges and two stomachs. (About 90 ×.)

abd. oes., abdominal oesophagus; end., endostyle; ep., epicardium; int., intestine; m-int., mid-intestine; per., pericardium; ph., pharynx; p-st., post-stomach; rec., rectum; st., stomach.



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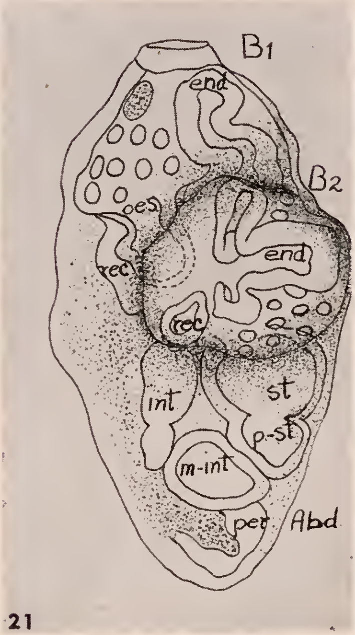
B1
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rec
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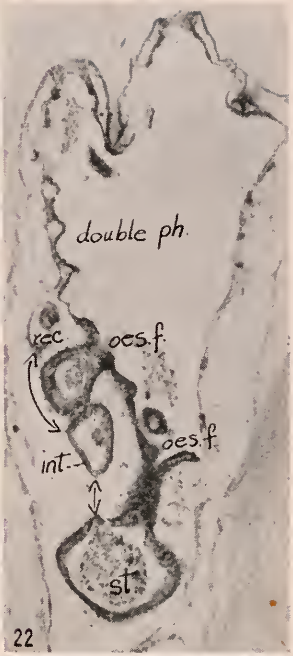
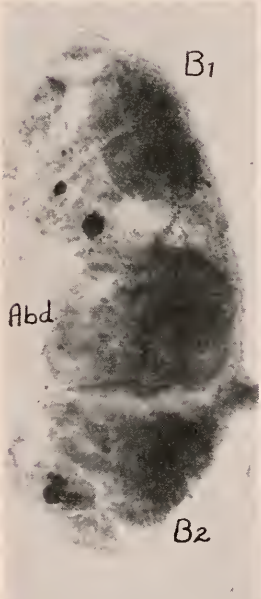
m-int
per
Abd.

21


double ph.
rec
oes.f
int.
oes.f
st

22



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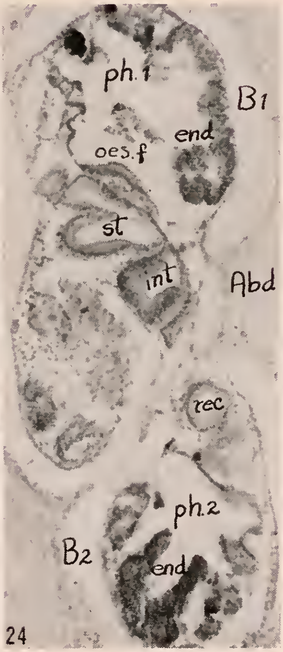
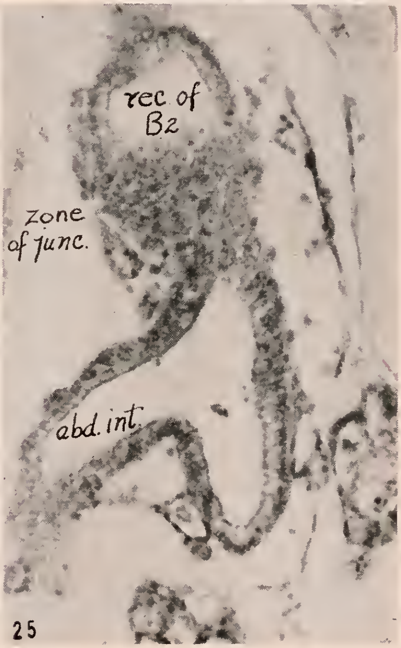

ph.1
B1
end
oes.f
st
int
Abd
rec
ph.2
B2
end

24


rec of B2
zone of junc.
abd. int.

25

FIGURE 20. Whole mount of two-day-old twin.
FIGURE 21. Camera lucida drawing of the same specimen to show its structure.
FIGURE 22. Longitudinal section of a five-day twin with fused pharynges and one stomach.
FIGURE 23. Whole mount of a two-day-old linear twin.

clouds of cells from dissociated fragments of the same organ. They move toward each other and close all gaps. Where the gap between parts is slight, the reintegration is quickly established; where the gap is wider, reconstitution is, obviously, effected more slowly. No attempt was made in the present study to reduce the organs to inconglomerate masses of minced miscellaneous organs. This degree of maceration was accomplished in the previous study to investigate the individual tunicate's capacity for reassembling itself from its own fragments. The problem of reintegration within individual organs in these triple combinations constitutes an accessory interest and merely a prelude to the main issue. Sufficient damage is inflicted on the organs to provide surfaces of injury between and among the three half zooids, this being a condition for their subsequent union. Organ reconstitution, however, does occur in each partial zooid to the extent that the portion of enteron contained in that half is restored before the dismembered pieces establish their integrity as Siamese twins.

Within a day, damaged and severed sections of stomach and intestine in the abdominal half are reunited into a continuous tract, and detached areas of the two pharynges in the branchial halves are reassembled into recognizable branchial baskets with all tissues in their relative locations (Figs. 15, 16, 17, 18). The oesophageal attachment, the so-called funnel, adjusts itself according to the length of its fragment, the variations in which depend upon the exact location of the knife in cutting between pharynx and stomach. The endostyle remains in a contorted condition through this early period of pharyngeal reconstitution (Figs. 6, 15, 16). Density of cell population in the endostyle and its position along the entire length of the pharynx predispose it to rupturing in the process of even mild maceration. There may be, therefore, frequent cases in which an endostylar fragment, in combination with the contiguous pharyngo-epipharyngeal wall, lies too widely separated from the main mass of pharynx for its reaggregation in the parent member. In the detached piece, then, the process of regeneration takes over and an accessory branchial sac is produced. The accessory one may attach itself to the parent pharynx and thus endow the composite animal with one double and one single pharynx or it may form an independent zooid. (Fig. 34).

The pharynx is so expansive an organ in its normal state that its reintegration is more slowly effected than that of the more compact loop of intestine in the abdominal segment. It continues through the first day in its crumpled condition while the wholly, or partly, dissociated pieces of each branchial component reassemble themselves into two complete pharynges. If a pharynx, because of variations noted previously in the cutting, retains only the oesophageal funnel or the funnel and a very short segment of oesophagus, the free surface is converted into the typical migrating cluster of cells, the direction of which, even at this early stage, may be toward the oesophagus of the abdominal segment (Fig. 8, lower half). If the pharynx retains a longer segment of oesophagus attached to its funnel, it shows,

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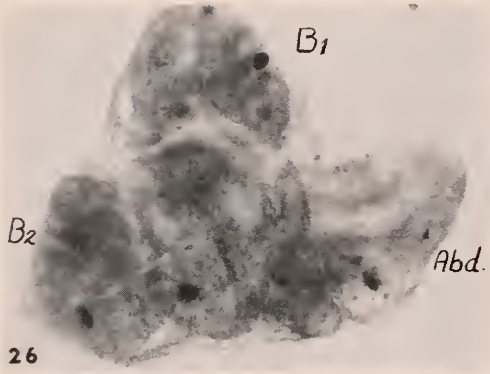
FIGURE 24. Longitudinal section of a linear twin of the same age.

FIGURE 25. Detail of the specimen in Figure 22, showing the zone of junction between the abdominal intestine and the branchial rectum. (About 90 ×.)

abd. int., abdominal intestine; end., endostyle; int., intestine; oes., oesophagus; oes. f., oesophageal funnel; per., pericardium; ph., pharynx; p-st., post-stomach; rec., rectum; st., stomach.



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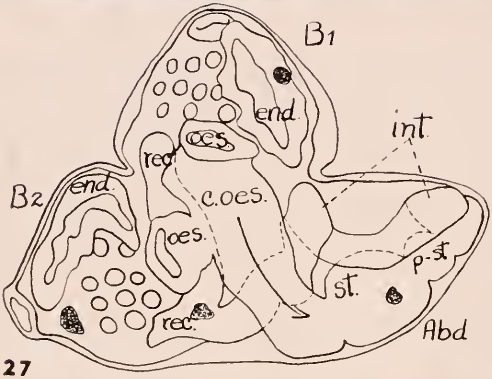
B1
end.
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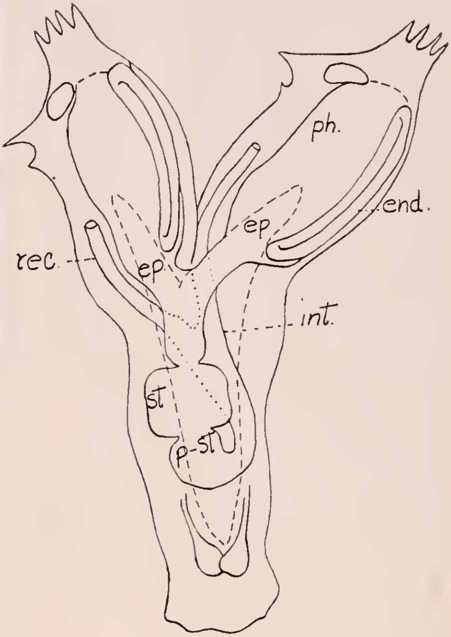
27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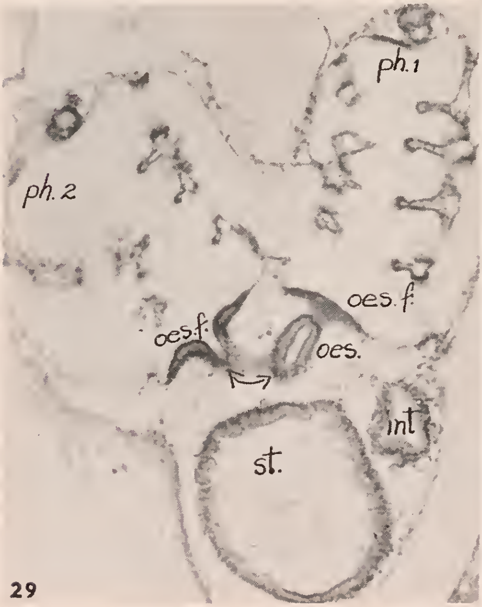
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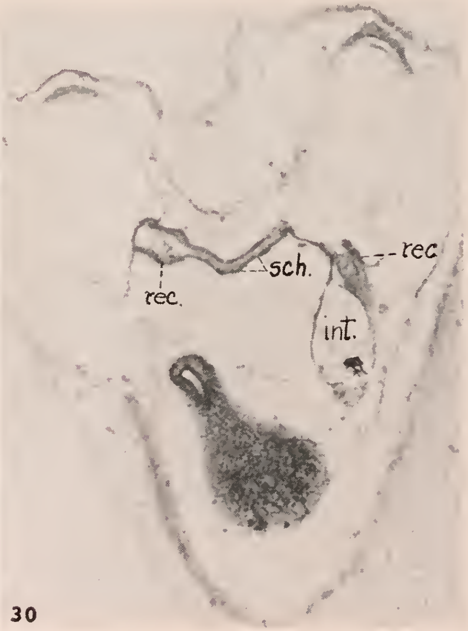
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Figs. 26–30.

from 9 to 12 hours, a definite orientation of its "fusion" bud in the direction of the oesophageal component of the abdominal region (Fig. 6, 8, upper half).

In these early stages of reintegration the behavior of epicardium and pericardium lacks significance. The epicardium emerges gradually from its compressed condition in the abdominal members to a more extended state as the digestive loop collects its pieces together (Figs. 6, 14). In composite organisms where the epicardium has been dissociated into two or more fragments, the fragments join into a single structure. Where any portion of epicardial epithelium is lodged, it retains the capacity of differentiating into an epicardium. There may thus be several epicardia which, eventually, fuse into one (Fig. 19).

The same degree of competence marks the epicardial epithelium with respect to the formation of a pericardium and heart. The primary heart may be destroyed in the procedure of mincing; if it is destroyed, then any fragment of epicardium close to an intestinal mass may differentiate into a heart. If the primary heart is not destroyed, it continues to contract, though empty of its normal contents, through the period of reaggregation and reconstitution. By reason of such competence of the epicardial epithelium, the pericardium as a structure lacks significance in the present study of triple fusion in tunicates.

The organ-parts of the half zooids show an immediate response in all tissues, within their specific histological categories, to re-establish their integrity; first, their individual organ integrity and, second, their organ-system integrity. These activities continue throughout the first day in all areas so that, by the end of one day of development, the triple zooids are, at least, partially fused to the extent that the epidermis is actively ensheathing the entire mass within a common mantle. There may remain several narrow gaps in process of closing and the surface is anything but smooth in contour (Figs. 13–18). The contents of the mantle still present a disordered appearance. It is less chaotic, however, than that of the initial stage. Surrounding the diversity of enteric fragments are loose remnants of caudal cells, if these have not been removed when the zooids are sectioned, bits of sensory pigment, patches of epicardium, and body cells. Cellular detritus occupies the open cavities of gastric and intestinal sectors and may be found in the space between body and tunic (Figs. 14, 18, 19). "Fusion" buds of all injured surfaces of organ-parts are in process of joining their homologous parts if they have not completed the union. In most combinations the related parts of the various members are separated by wide distances, histologically speaking, and the process of reaggregation has only begun in pharynges and digestive loop by the end of the first day (Figs. 13–18).

The second and third days of development are marked by progress in reintegration of all organ parts and expansion of all areas toward their normal proportions. The epidermis, though incomplete in spots, provides the animal with a more unified

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FIGURE 26. Whole mount of four-day twin.

FIGURE 27. Camera lucida drawing of the same specimen.

FIGURE 28. Diagram of the structure of Y-shaped Siamese twin in its functional stage of development.

FIGURES 29 and 30. Longitudinal sections through two regions of a Y-shaped rectal fragment. (About 90 ×.)

c. oes., common oesophagus; end., endostyle; ht., heart; int., intestine; oes., oesophagus; oes. f., oesophageal funnel; ph., pharynx; p-st., post-stomach; rec., rectum; sch., shuttle rectum.

appearance, and over-all expansion of tissues from their contracted state enables the body to occupy more fully the tunic cavity of the host (Figs. 20, 21, 22, 23, 24).

The oesophageal stub of each branchial sac extends farther in the direction of the homologous oesophageal stub of the abdominal member. In the composites that effect a Y-shaped union, the branchial sacs unite either separately or jointly with the stomach; that is, the two funnels may fuse and then combine with the single oesophagus of the stomach (Figs. 26, 27, 28, 29) or one funnel may engage the gastric stub and the second funnel reconstitute an independent oseophageal connection with the stomach (Fig. 22). Since the chance union of the branchial baskets in these forms ranges from a Y with widely diverging limbs through parallel alignment of pharynges to partial fusion of them, with two sets of siphons projecting at various angles from the fused mass, the paths followed by the oesophageal stumps also vary in accordance with the relative positions of the homologous parts.

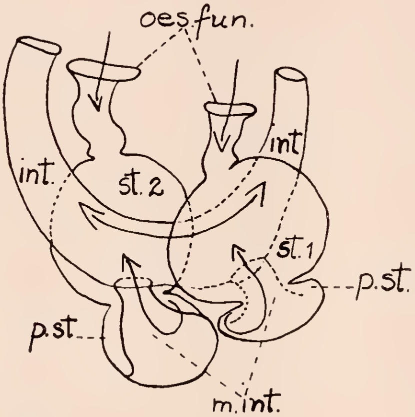

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FIGURE 31. Diagram outlining the channel of traffic in a form with two stomachs and one intestine.

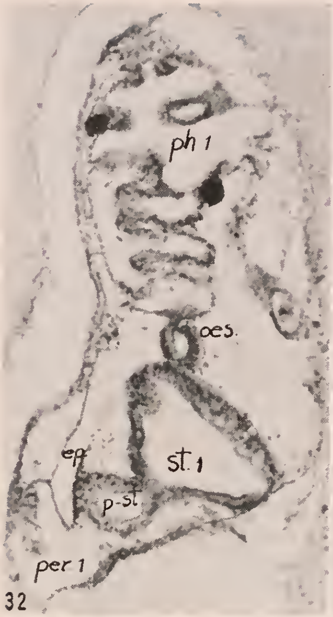
Each dissociation depending, as it does, on the delicate conditions of the zooid's sensitive response to cutting and the critical control of a micro-knife, within the distance of 0.1 mm., it is evident that parts of the stomach may be included in the branchial member along with the other usual constituents of that member. In such cases the partial stomach reconstitutes itself and the secondary product associates itself, at its posterior end, with the intestinal loop beyond the primary stomach. The end product in this composite possesses two stomachs, two oesophagi and one intestine. Figures 32, 33, 35 and 36 show the systemic organization of twins with two stomachs and a single intestine. Stomach 1 is the primary one, that is, the one contributed by the abdominal fragment. Stomach 2 is the secondary one which originates in a gastric remnant introduced by a pharyngeal member. The loop of intestine adapts itself to two stomachs by retaining its original relationship with the primary stomach and then establishing a secondary connection with the post-stomach of the branchial fragment, thus converting the tube into a crescent-shaped structure reaching from the double stomach toward two terminal apertures. In these twins, each oesophageal-stomach unit follows its independent pathway into the intestine which, then, diverges toward the rectum and anus of the two branchial components. Figure 31 is a diagrammatic reconstruction of the double gastric circuits of traffic into the common intestine and, ultimately, into the separate anus at the base of each atrial siphon. Despite the proximity of the secondary stomach to the unengaged rectal segment of the same branchial sac, the intestinal union is established between abdominal intestine and this fragment in accordance with the linear gradient of the digestive tract.

In the Y-shaped triple fusions in which a gastric fragment is not included in the branchial member, there is a single stomach serving both pharynges (Fig. 22). The alimentary tract in such twins consists, therefore, of two oesophageal funnels which relate the pharynges to the common stomach. The post-stomach, mid-intestine, and intestine may lie close to one rectal fragment and far removed from the second one. The digestive channel, in such cases, is completed without including the distant one in the union. Figure 30 demonstrates the reaction of the isolated rectal fragment to its histological situation. It produces a bud of cells which directs its growth toward the homologous rectal remnant of the sister branchial member. The bud differentiates into a shuttle channel which opens immediately adjacent to the anus in the atrial chamber of the connected alimentary tract. The traffic circuit of the enteron in these reaggregations consists of double pharyngeal chambers converging to a single oesophagus and proceeding through a common channel from that point to the anal opening in one of the branchial baskets. From that site, passage of excreta may continue directly to the atrial siphon of the branchial member involved or it may be deflected through the shuttle rectum to the siphon of the twin member on the opposite side (Fig. 30).

In many twin composites, the two pharyngeal regions are so close together that they fuse along their longitudinal axes and form a double pharynx. All structures remain duplicated, including the oesophageal funnels. In the five-day-old twin, represented in Figure 22, one funnel has and one has not made its connection with the common stomach. The intestinal segment passes behind the unconnected funnel to establish contact with the rectal fragment. The intestinal epithelial patches join with the oesophageal stub to the extent that their walls form a surface union in passing. There is functional fusing only between the intestine of the abdomen and the rectal remnant of the branchial sac. The cluster of cells reproduced in Figure 25 shows the union being established between the two intestinal fragments. Unions are also being completed between macerated fragments within the members. Stomach and mid-intestine of the same individual are reassembling themselves into an integrated whole at a point posterior to this intestinal junction between organs originating in two different individuals.

The epicardium reaggregates its parts into a unified structure by the same process of fusion of homologous parts. In Figure 19 the two constituent fragments can be seen in the act of joining together to form a single epicardial cavity. Body cells that supply nourishment for these tissue activities are concentrated at the sites of all junctions.

Zooids that form a longitudinal axis of Siamese twin, with central abdomen flanked by pharynges, present interesting patterns in morphological adaptations to tissue affinity. The stomach occupies various positions in the limited area between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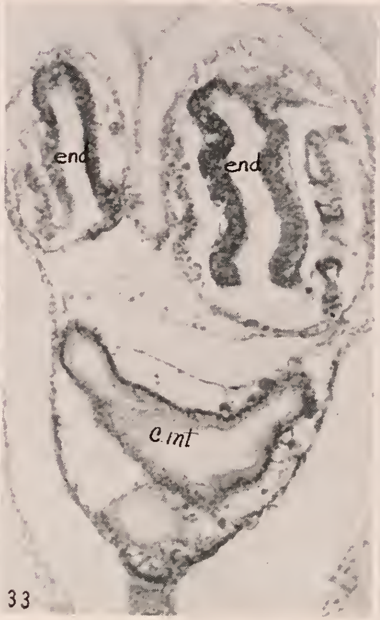
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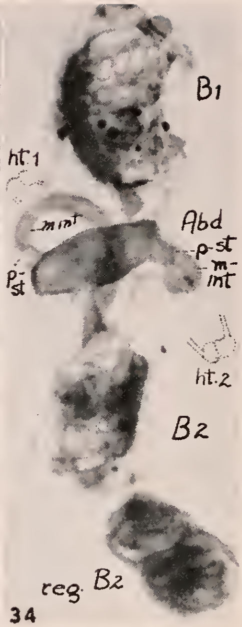
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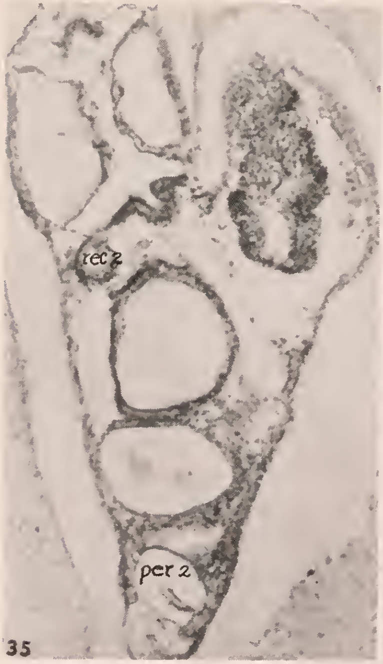

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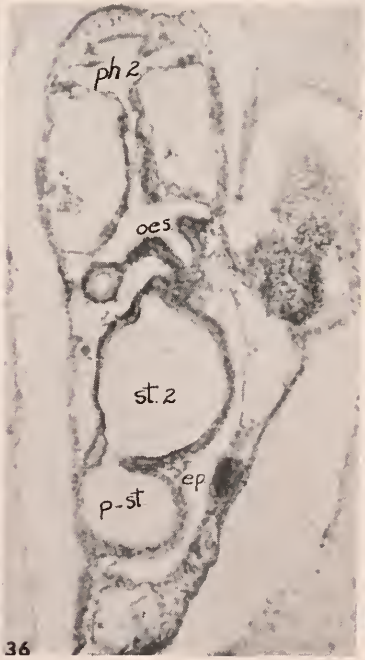
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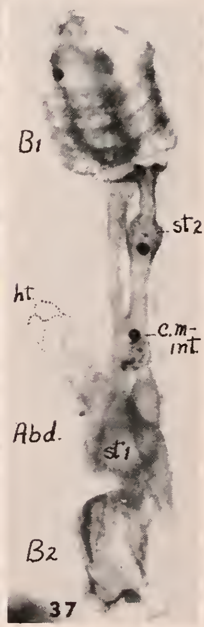
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FIGURES 32, 33, 34 and 35. Successive sections through a five-day-old twin with two stomachs and one intestine.

FIGURE 36. Whole mount of a five-day-old Siamese twin with a horizontal stomach.

FIGURE 37. Whole mount of a five-day-old twin with primary and secondary stomachs and a common mid-intestine. (About 90 ×.)

the pharynges. Figure 37 shows the stomach lying in close proximity to one pharynx with which it unites by fusion of the pre-gastric oesphagus of the abdomen with the funnel of that pharynx. The second pharynx is farther removed from the stomach. It engages in activities of both aggregation and regeneration in establishing contact with the central digestive organs. Its oesophageal funnel regenerates a tubular channel which grows toward the mid-intestine of the primary tract with which it fuses.

The rectal fragment in each branchial donor effects association with the common mid-intestine by processes that seem to be determined by proximity between the members. The rectum lying close to the severed intestine joins it through the complementary fusion buds that characterize the reunion of all detached homologous tissues in similar macerated zooids. The more distant rectum moves by extension and cell proliferation toward the mid-intestine of the common stomach and fuses with it. This intestinal limb, like the gullet of the same pharynx, is partly the product of regeneration. The channel of traffic in this type of reaggregated *Amaroccium* now follows the course of a chiasma, with oesophageal-gastric limb crossing intestinal-rectal limb between the two pharynges (Fig. 37).

Within five days in such a twin the long oesophagus differentiates an accessory or secondary stomach at the appropriate distance from the pharynx. The two regenerated members of the composite thus establish a second series in the normal linear sequence of digestive organs without disturbing the chiasma through a common mid-intestine. Digestive traffic may proceed in either direction toward either terminal siphon from the primary stomach. Black pigment from both sensory vesicles may be seen in any region of the tract. Pigment of otolith and eye is one of the substances to be eliminated early when passage is cleared in the reconstituted digestive canal of all reaggregates of these zooids whether they be simple or multiple.

There is a single heart in the twin described, at least on the fifth day of reconstitution. It occupies the position it held in the abdominal mass. Fusion of the three masses has settled its location to a position intermediate between the two pharynges, or in the center of the digestive field.

If both branchial baskets, in a tandem arrangement of twin, are equally close to the abdominal member, another adaptation in spatial relationship occurs. The oesophageal stub of the single stomach unites with the funnel of the pharynx in its immediate field. The intestine establishes its connection with the rectal fragment of the same pharyngeal component. The stomach widens horizontally and the funnel of the twin pharynx sends its bud of cells toward the stomach on the side facing it. The corresponding posterior side of the stomach regenerates an intestinal limb which meets the bud of cells directed from the rectal fragment in the same twin atrium. A second axis of polarity is set up on this side of the complex organism in keeping with and, probably, induced by the close proximity of another pharynx to the stomach. The pharynges, in this case, form a straight angle with each other and a right angle with the common stomach. Each one assumes its proper entrance and exit according to the axial polarity that obtains between these two organs in zooids

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c. int., common intestine; c. m-int., common mid-intestine; end., endostyle; ep., epicardium; ht., heart; oes., oesophagus; per., pericardium; ph., pharynx; p-st., post-stomach; rec., rectum; st., stomach.

of tunicates. The original axis of polarity is preserved between one pharynx and abdomen; a similar axis is set up between the other pharynx and abdomen. The basic pattern of spatial relationships is either retained or acquired on both sides: oesophageal funnel opens toward the dorsal region of the pharynx on its right and the intestine passes to the rectum on the left of the stomach. Identification of the primary intestine in such four-day twins is ascertained by the presence of the post-gastric and mid-intestinal valves. These have not yet differentiated in the regenerated intestine. The double axis of polarity is further marked by the development of an accessory heart formed by the epicardium in the area of the secondary intestinal loop. Figure 34 is a photomicrograph of a longitudinal twin with the stomach riding transversely between the two pharynges. The print shows a fragment of one of the pharynges which, having failed to become incorporated into its parent member, is regenerating another zooid. Such fragments form whole zooids independently of the histological activities in the reconstituting twins. A similar regenerative process occurs in fragments of a single *Amaroecium* when they come to be located at distances from the main mass of tissues into which an organ, or region, is macerated, or when a capsule of epidermis separates any one part of an organ from the remaining fragments.

Final topographical relationships among the triple components are unpredictable: a natural consequence of the conditions necessary for inserting the pieces separately into a tunic, mincing them moderately to insure contact of injured surfaces and, finally, pressing a plug of tunicin against the pliant mass to hold it intact. There are numerous minor variations, therefore, in the final appearance of the composite organisms. There is, however, a uniform pattern of behavior among the tissues themselves that is affected only incidentally by the shifting of their major component parts.

In the abdominal member, the long segment of intestine re-establishes itself as an integrated organ from the lesser or greater number of parts into which maceration has reduced it. The valves that differentiate it into post-stomach and mid-intestine are present and all the detached patches become incorporated into a continuous alimentary tract: continuous insofar as the abdominal member is concerned; it may still be in process of effecting union with its twin homologous organs in the two branchial members, oesophageal funnel and terminal rectum. Each rectal remnant directs its fusion bud, or its proliferating bud, toward the intestinal surface. When one rectal fragment is closer to the rectal fragment of its twin pharynx than to the intestinal tissue, it selects the shorter path to its corresponding organ-part. The final association resolves itself into a bifurcated intestine (Fig. 33) or a single canal with a shuttle branch connecting one detached anus with the other integrated anus (Fig. 30). These unions are completed, or about to be effected, in 5-day aggregates but their associations are indicated by the buds of migrating cells that form at each dissociated surface of intestine, rectum and oesophagus in two- and three-day composites.

In the branchial members, the walls of branchial sacs continue to extend endostyles and patches of gill clefts into more expansive surfaces. Gaps in epidermis and respiratory epithelium are closed gradually by migratory action of the bordering cells as well as by cell proliferation proceeding at the normal tempo in the uninterrupted areas which, therefore, contribute cells, indirectly, toward supplying the gaps.

After five days of development, the architecture of the composite tunicate is fully established. All tissues are united according to their kinship through embryonic origin and histological differentiation. The branchial sacs, in Y-shaped or tandem association, are in functional union with the abdominal organs. The epidermis is an uninterrupted sheath enclosing the composite animal. The heart is providing the mechanical force for circulation of the body or "blood" cells through the tripartite body. The nutrition they carry has been retained through the time of reconstitution or obtained from the food passing through both oral siphons in those Siamese twins whose siphons are already functioning. In those "twins" having two hearts, both of them are in functional activity.

All early stages in both whole mounts and sections show the disordered appearance of the Siamese twins when cellular detritus is being discarded into the general tunic cavity through gaps in the epidermis. The varying amounts of tissue destroyed during mechanical dissociation determine the length of time required to clear up the tunic cavity. The black masses visible in all whole mounts of three-, four- and five-day forms are the remnants of sensory pigment that are being eliminated through the digestive tract as soon as its parts are reaggregated into a complete system. Throughout the period when the tissue components are reassembling themselves into a double monster, masses of necrotic cells are found in the lumina of all regions of the dissociated digestive tract. Ciliary movement is not interrupted either by sectioning the zooids into halves or mincing the three halves together in the host test, and the loose bits of tissue debris are drawn into all the disconnected segments of pharynx, stomach, intestine, and oesophagus. When body form is recovered, these masses are the first contents to be expelled through the atrial siphons. Sensory pigment follows and, by that time, both oral siphons are open for feeding.

The composite twins were not studied in this investigation beyond the acquisition of feeding activities. Having reassembled the various numbers of tissue components into their original organ status, and having joined the reconstituted organs of three contributing individuals into one integrated organism of Siamese twin character, with all regions aligned in the axial organization from which they were separately disjoined, the reaggregated functional zooids were considered a natural terminal stage in the present study.

DISCUSSION

The capacity of cells to recognize their own kind in a scrambled mixture; to combine selectively with them in preferential associations; and to reject any association with non-matching cells is now a well established reality. Numerous studies have contributed significantly to the mass of information available in this field. Because of the evasiveness of the issues involved in the problem of specificity of tissues, the studies tend to be descriptive rather than analytical. The question has many facets and experiments have been designed to investigate numerous aspects of it. Moscona (1960) presents one such analytical approach in his report on the significance of the extracellular material matrix (ECM) as a substitute ground substance which, in culture media, provides the cell population with a "controllable microenvironment." He favors the hypothesis that the matrix formed in tissue cultures exerts an orienting influence on cell movements and a binding effect on the cells in their structural configurations. He, thus, assigns to the matrix the dual function of cell-bonding and transferring information.

Wilson (1908, 1910, 1911) and Galtsoff (1925) first recognized the specific attraction of dissociated cells in their classical works on sponges and hydroids. Their interest was focused on the behavior of cells dissociated and retained in the natural environment of the animals, not in tissue culture. Another report in this early period involved entire organisms at the chordate level. It is the account of Harrison and Pasquini (1930) on the Mediterranean tunicate, *Clavelina*. In their unique "piggy-back" experiment they were concerned with the demonstration of persisting polarity rather than tissue affinity. They described fusion between two segments of branchial baskets in which the axes of polarity were retained through the process of dedifferentiation characteristic of this tunicate, and through the subsequent period of reconstitution. Their line drawings of the reconstituted "monsters," interpreted in the light of the results reported in the present paper, indicate that the tissues in the grafted segment of *Clavelina* fused with matching tissues in the host zooid. The observations, however, do not refer to these morphological phenomena. A more recent study (Scott, 1959) has analyzed the specific reactions of matching tissues in a simple straightforward case of disaggregation of one complete animal, the tunicate, *Amaroecium,* and its reconstitution into a functioning zooid. Fragments of macerated organs reassemble themselves into their respective parts and then align themselves into a complete digestive system in rigid accordance with their former axes of polarity. Within a period of five days the reconstituted zooids are feeding. In such an experiment the tissues are in fully differentiated adult condition with the exception of the stomach in which the glandular crypts have not yet developed.

The regions of the digestive tract are distinguishable from each other by reason of the simple histological patterns that characterize epithelial cells: position of nucleus, relative depth of cell ranging from deep columnar in oesophagus, stomach, post-stomach and mid-intestine, to cuboidal in intestine to squamous in pharyngeal wall. These histological tags and the unique circumstance whereby the dissociated animal can be confined within the natural (and supporting) environment of its own tunic endow *Amaroecium* with particularly advantageous traits for the study of tissues in expressing their specificity and affinity. It is an excellent instrument for combining experiments on tissue-to-tissue attraction and the persistence of axial polarity, not merely in organ stability, but in the indestructible integrity of the individual.

These same traits are employed in the present investigation which adds another dimension to the previous study. The individual zooid, macerated in its tunic, demonstrates the behavioral patterns among tissues of similar histogenetic character which originate by processes of differentiation and organogenesis within a single embryo. The mincing together of three halves of three separate individuals, and their subsequent reassociation to form harmoniously functioning Siamese twins, reaffirms several properties shared by homologous tissues whether they originate in one embryo or find themselves in the unnatural situation of sharing a host tunic with fragments of several zooids: they join only with their own specific histological relatives; they regulate by regenerating any deficiencies among the regions that are required for proper association of organs when one set of organs is doubled; they retain the axiation that marked their previous alignment.

The regulatory process of induction also manifests itself in some of the tandem alignments of twins. The regenerated oesophageal tube produces a secondary stomach at the site normally occupied by a stomach in the undisturbed zooid. Induction in this case does not agree with Grobstein's (1954, p. 234) definition of it as a "developmentally significant interaction between closely associated but dissimilarly derived tissue masses." The tissue is a canal of digestive epithelium regenerated from the dissociated funnel of that pharynx farther removed from the single stomach than its juxtaposed twin pharynx with whose homologous funnel the gastric oesophagus joins. The tissue is, therefore, of the same embryonic origin as the pharynx and is, in fact, derived from it. In the typical pattern of reunion of these triple components, such differentiation into regional organs does not occur. The process is a straightforward one of meeting and fusing of homologous tissues. The appearance of an incipient stomach in the unusually long oesophagus can be explained only in terms of the inductive influence of the pharynx acting on the tissue associated with it which, in the course of embryonic development, does differentiate a stomach at that site.

It may be considered an induction in the sense of Wigglesworth's (1959) theory of a "field" in which the process of differentiation follows a gradient set up or determined by the interaction of "inductor" substances with a substrate. He refers to an undifferentiated substrate, but undifferentiated in the sense of providing a ground matrix of fundamental tissue which, by reacting with the "inductor," initiates differentiation. In this case the fundamental tissue is from oesophageal epithelium, a basic type of endodermal epithelium, and it differentiates into gastric epithelium, a more specialized kind of tissue. The substances, of whatever nature they are, have effective pathways to follow in setting up a center for their activity; they may be carried in the lumen of the canal, in the body cavity or diffused through the cells themselves.

The specificity which marks all tissues in their fusion reactions conforms, also, to the principle of histological clannishness which Wigglesworth (1959) finds in the tissues of *Rhodnius*. Digestive epithelium that is differentiated into oesophageal funnels unites only with gastric oesophagus or with the superior surface of the stomach toward which the oesophageal funnel of one of the branchial baskets grows and with which it combines. If the two oesophageal funnels lie in close proximity, there is no regeneration. The two stubs fuse and the single oesophagus of the abdominal member unites with the fused pair. Regulation is determined, apparently, by proximity of matched remnants; selection, or preference, is determined not only by the fact of differentiation into digestive epithelium but, also, by reason of the degrees of differentiation along the axis that marked the tissue in its former particular niche in the digestive sequence. The fragment of rectum that is always included with the branchial basket directs its fusion bud toward the intestine of the abdominal portion, not to the stomach or oesophagus. If the rectal fragment of one branchial member is closer to the corresponding fragment in the second branchial member, then a shuttle junction is established between these two terminal sections of rectum. It serves no functional need. Elimination of waste is effected through the intestinal loop that establishes contact between the common stomach and one of the terminal fragments. The rectal epithelium of the more distant piece, however, can satisfy its predetermined character only by uniting with its matching counterpart. Weiss and Taylor (1960) refer to this as "autonomization" or "self-organization" and they consider it as a property of a developing organism.

In this case, however, it is a property of adult tissue and totally removed from any implication of being associated with induction. It is the interaction between tissues coded, by their differentiation, to the same cues. Weiss (1947) considers the cues as chemical characteristics of contact surfaces. This response between two detached homologous fragments of rectum characterizes the behavior of all regions of the digestive tract during their reconstitution. The degree of specificity is refined to the point of region-to-region attraction within the framework of an uncomplicated system consisting of a linear series of organs of common origin and differentiated from each other only by slight variations in a continuous simple epithelium. The shuttle segment of rectum is merely one case in point. Such specific matching of tissues marks all other regions of the tract and the epicardium as well. The epicardium presents a deeper enigma in its behavior in view of the fact that its cells retain their embryonic status throughout larval and adult life until the time of budding, when each segment of epicardial tube differentiates into a complete digestive system. In the absence of differentiation beyond the basic possession of digestive epithelial potency, fragmentation might be expected to trigger the developmental mechanics of differentiation in detached patches of this tube. Constriction into segments by the epidermis initiates the process of such developmental activity in the normal period of budding. Experimental constriction of the short epicardium in early adult stage induces it, prematurely, to form a small zooid.

By mildly mincing the three half zooids into a compact mass, the epicardial sections are further disrupted into numerous disorganized fragments. The epithelia of the fragments meet, fuse, and reconstitute a common epicardium for the Siamese twins. Attraction between like tissues, therefore, dominates over the inherent capacities for both regeneration and differentiation in these tunicate tissues. Whatever the factors are that enable one tissue to recognize its kind, they operate at a higher level of determination than the (1) properties that enable tunicate fragments to reconstitute missing parts through their facile regenerative activities, and (2) the virtuosity of the epicardium in producing, with epidermal aid, a complete zooid. Specificity in tissue affinity in these primitive chordates is associated with a strong system of axial gradients and the combined set of potencies insures reintegration of a composite scramble of tunicate parts from several zooids into a monstrous but harmoniously functioning individual. The character that is impressed on tissues through the process of differentiation is persistent to the point of regulating the types of aggregation in dissociated masses of tissue whether these masses originate in one or in several individuals of the same species.

The nature of the cues which these tissues follow in re-establishing their integrity in orderly steps from organ through system to organismic level evades analysis. Wilde (1958) suggests that the characterization of coding substances may be nucleic acid or protein or both and he postulates patterns of electron flow as the chemical process whereby information may be transferred from one kind of cell to another. He locates the key to cellular differentiation and specificity at the molecular level. Mirsky and Allfrey (1958) review various researches supporting the logical conclusion that the nucleus, specifically DNA in the nucleus, controlling as it does, through RNA, protein synthesis in the cytoplasm, must be responsible for the specificity of the cell. DNA participates in the synthesis of nucleolar RNA which may pass into the cytoplasm where RNA, in turn, may direct the synthesis of proteins that differentiate one specific kind of cell from another. The same coding, therefore, that is transferred from DNA to the cytoplasm through the medium of nucleic acids in the expression of genetic traits may be responsible for the cues that direct tissues to unite and fuse only with their own homologous types.

SUMMARY

1. Young adult zooids of *Amaroecium constellatum* were divided into branchial and abdominal halves.

2. Two branchial halves and one abdominal half were inserted into the tunic of a two-week-old zooid from which the occupant had been evicted.

3. The three halves were mildly macerated in order to bring their injured surfaces into contact.

4. The epidermal mantle of the three half zooids, by the spreading action of its cells, enclosed the triple members in a common epidermal mantle.

5. The organ-parts in each contributing half ressembled their fragments into complete organs.

6. Union of organ-parts was effected only between tissues of matching histogenetic character.

7. When the dissociated organs re-established their integrity, the organs of each half united with their homologous members in each other half.

8. The re-establishment of organs into systems followed the system of axial gradients that characterize these tunicate zooids.

9. Within five days, three halves of three zooids reaggregated their constituent parts into functioning Siamese twins demonstrating various patterns of recombination.

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OSMOTIC BEHAVIOR IN AN INTERTIDAL LIMPET, ACMAEA LIMATULA[1]

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Marine invertebrates generally are considered to be poikilosmotic. Varying degrees of ion and/or volume regulation have been demonstrated in decapod Crustacea, polychaetes, and a few other selected groups. Little information is available on the osmotic behavior of molluscs. Bethe (1934) has shown that in 75% sea water the nudibranch *Doris* possesses essentially no volume regulation and loses salts, in the form of chloride, over a 48-hour period. In the tectibranch, *Aplysia*, Bethe (1929, 1930, 1934) concluded that the response to 75% sea water is an initial weight increase, and a subsequent loss in weight as salts and water are lost from the body. But van Weel (1957) reported that *Aplysia* could tolerate only 95% sea water and that further dilutions seriously damaged the animals. *Onchidium*, a marine pulmonate, swelled in dilute sea water but regained its original weight after return to normal sea water, demonstrating that little salt was lost (Dakin and Edmonds, 1931). No data appear to be available for prosobranch gastropods.

In intertidal prosobranch gastropods, volume changes in response to increased salinities seemingly cannot be separated from volume changes due to desiccation brought about by exposure. This problem is particularly acute in species with a relatively wide intertidal vertical distribution. All prosobranchs possess an extra-visceral space between the shell and soft parts. Water retained in this space serves as a jacket around the head, ctenidia, portions of the visceral mass and foot, and may serve to retard desiccation. Also, in a study of water relations in the limpet, *Acmaea limatula* Carpenter, Segal (1956) suggested that the extra-visceral space may have an osmoregulatory function.

The object of this study is to determine whether the limpet *Acmaea limatula* is capable of osmotic regulation over a range of salinities, and to determine whether extra-visceral water serves (1) an osmoregulatory function, *i.e.*, maintains an ion and/or water gradient between the blood and the external medium, and (2) as a temperature buffer.

MATERIAL AND METHODS

Acmaea limatula used in this study were collected from the following three geographic locations: Dike Rock, immediately north of Scripps Institution of

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Oceanography, La Jolla, California (32°, 52′ N Lat.; 117°, 15′ W Long.); northeast side of Punta Banda (Todos Santos Bay), Baja California (31°, 44′ N Lat.; 116°, 42′ W. Long.); southwest side of Punta Banda (Papalote), Baja California (31°, 44′ N. Lat.; 116°, 43′ W Long.). Dike Rock is on the open coast, with a mean inshore summer temperature range from 19° to 21° C., mean winter temperature, 15° to 17° C. The northeast side of Punta Banda is a protected warm water coastal bay with a summer inshore temperature range from 21° to 25° C. The southwest side of Punta Banda is a cold-water, upwelling open coast area with a summer inshore temperature range from 14° to 16° C. During the winter months the mean difference in inshore water temperatures between the two sides of Punta Banda is less than during the summer months (Todos Santos Bay is only 1° to 3° C. warmer). Mean temperature values for summer and winter for Punta Banda were obtained from Hubbs (personal communication).

Salinity experiments

Animals collected at Dike Rock were returned to the laboratory and placed directly into the experimental salinities, 25, 50, 75, 125 and 150% sea water, at a cold room temperature of 20° C. All animals were kept in the dark, not fed and maintained in plastic containers (about 3.5 liters sea water each), approximately 30 animals per container. Animals collected from Punta Banda were placed into salinities of 50 and 150% sea water. Required temperature (20° C.) was maintained in a dry ice box.

Salinities above 100% sea water were obtained by freezing normal sea water. The concentrated sea water was then diluted to the required salinities with distilled water. Salinities below 100% sea water were obtained by adding distilled water to normal sea water. All field and experimental salinities are expressed as percentage sea water, based on a standard sea water, 33.70‰ salinity, 18.65‰ chlorinity at 20° C. Salinities were determined on a 1000-cycle conductivity bridge calibrated to the standard sea water noted above.

Blood was sampled by making an incision through the ventral surface of the foot into the ventral sinus. Blood was collected in 0.4-mm. (I.D.) tubes, sealed with Nevastane grease and quick frozen on dry ice. Mucus, residual water and debris were removed carefully from the foot before the incision was made. Blood of animals from Dike Rock was sampled at three, 24 and 48 hours after immersion at the various salinities. Blood of animals from Punta Banda was sampled three hours after immersion. Blood from control animals (100% sea water) for both Dike Rock and Punta Banda experiments was obtained immediately upon removal of the animals from the normal habitats. The value determined for the control animals served as a baseline, and this was essentially 100% sea water. A modified method for melting point determination, as described by Gross (1954), was used to determine total osmotic pressure.

Field desiccation experiments

At Dike Rock, experiments were conducted on July 13 and July 15, 1960. On July 13 approximately 150 animals were collected, randomly divided for individuals and size and placed on grey lava and grey sandstone above the high tide level for that day. All limpets attached immediately. Blood was collected in the same manner as described for the salinity experiments. Extra-visceral water is the volume of water which is contained within the mantle cavity and surrounds the visceral mass and the foot. At the time of collecting animals this extra-visceral water is essentially sea water, and is in equilibrium with body fluids. Extra-visceral water was collected by thoroughly damp-drying the limpet, then gently applying pressure to the foot. Both blood and extra-visceral water samples were collected at the same time and handled in the same manner. Blood and extra-visceral water samples from the experimental animals were collected at two-hour intervals over a six-hour period. All samples were frozen immediately.

On July 15 approximately 150 animals were collected, half of which were placed on grey sandstone. The remaining 75 animals were shaken and blotted to remove as much of the extra-visceral water as was feasible. Then, they were placed near the first group. Blood and extra-visceral water samples from the experimental animals were collected at two-hour intervals over a six-hour period.

On July 27 approximately 150 animals were collected from Papalote (Open Coast), Punta Banda and returned to Todos Santos Bay. These animals were marked and placed at the level occupied by individuals of the Bay population. On July 28 approximately 100 animals each of the Todos Santos Bay and Papalote populations were removed from the bay and placed on light sandstone above the high water mark for that day. Blood and extra-visceral water samples from the experimental animals were collected at two-hour intervals over a six-hour period. Samples were frozen and returned to Scripps for analysis.

For all field experiments blood and extra-visceral water samples from control animals were taken at the time the experimental animals were removed from the intertidal region. These samples served as the baseline values, namely 100% sea water.

Rock surface and animal temperatures were obtained with a thermistor probe.

Weight change experiments

To determine the change in water content, expressed as per cent body weight, twenty animals each from Dike Rock were placed in 50, 100 and 150% sea water in the dark, at 20° C., for a period of 24 hours. Total damp-dried wet-weight was determined before and after animals were placed at the different salinities. After 24 hours, soft parts were removed from the shell and both soft parts and shell were weighed separately. Shell and soft parts were dried to constant weight at 110° C. for 24 hours and dry-weight of each was determined.

All animals used for the salinity, field desiccation and weight-change experiments were collected from ecologically similar intertidal locations; they were submerged at approximately a 1.5-foot tide.

RESULTS

Salinity experiments

The data shown in Figure 1 for Dike Rock animals demonstrate conclusively that *Acmaea limatula* does not regulate osmotically to either low or high salinities. Isotonicity is reached within 24 hours over the range of salinities. After this

24-hour period the concentration difference between blood and external medium was approximately 5% sea water, a gradient to which no significance is attached. At all of the salinities, except 25% sea water, no natural deaths occurred within 48 hours. Within six hours limpets held at 25% sea water were extremely swollen. These animals were all dead by 24 hours. Body fluid concentrations of control animals for all salinity experiments at the three collecting areas were determined, and differed only slightly from 100% sea water. No biological significance is attached to these differences. Animals in 125 and 150% sea water were isotonic with the media in three hours; in 50 and 75% sea water they were not. Furthermore, the lower the external concentration, the greater the gradient between blood and external medium. For example, at three hours, animals in

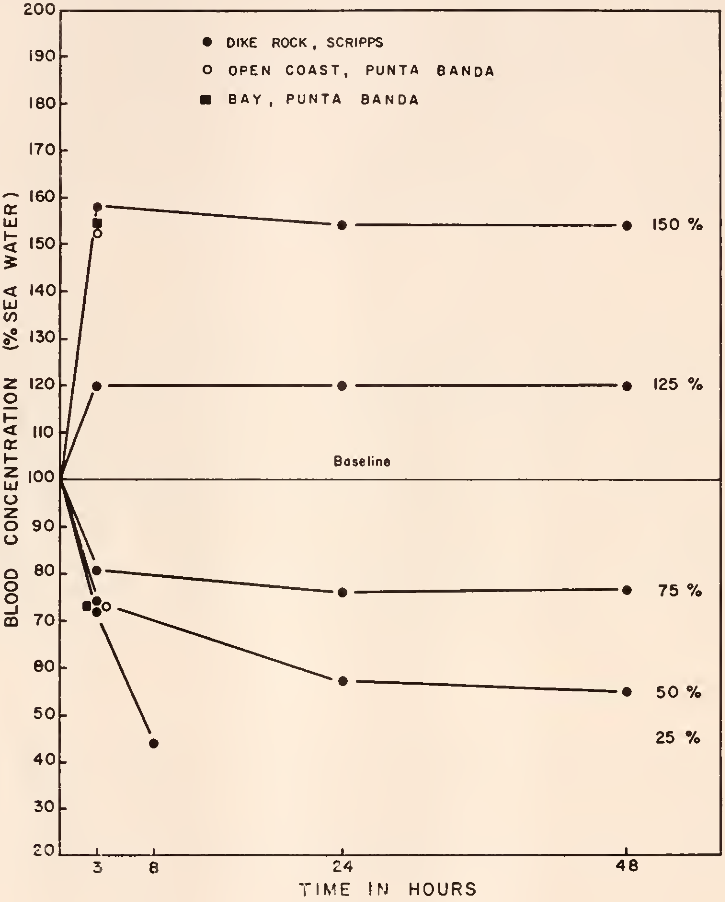

FIGURE 1. Change in blood concentration, expressed as per cent sea water, as a function of time, in hours, for three populations of *Acmaea limatula*. Each point is the mean value for at least ten animals. Line at the 100% sea water value represents the baseline for the three populations. Experiments were run at 20° C.

75% sea water have approached 8% sea water of being isotonic with the medium; in 50% sea water, 48%; in 25% sea water, 70%.

Examination of Figure 1 shows a slight hypertonicity at 150% and 50% sea water, and hypotonicity at 125% sea water after 24 hours. It could be suggested that this demonstrates a weak hyper-osmotic regulation. However, these points represent an average, and the variability of individual blood concentrations would seem not to permit this conclusion, based on these data alone. Further, at the two higher salinities, blood concentration is absolutely higher in one and lower in the other, showing no constancy toward hyperosmotic regulation.

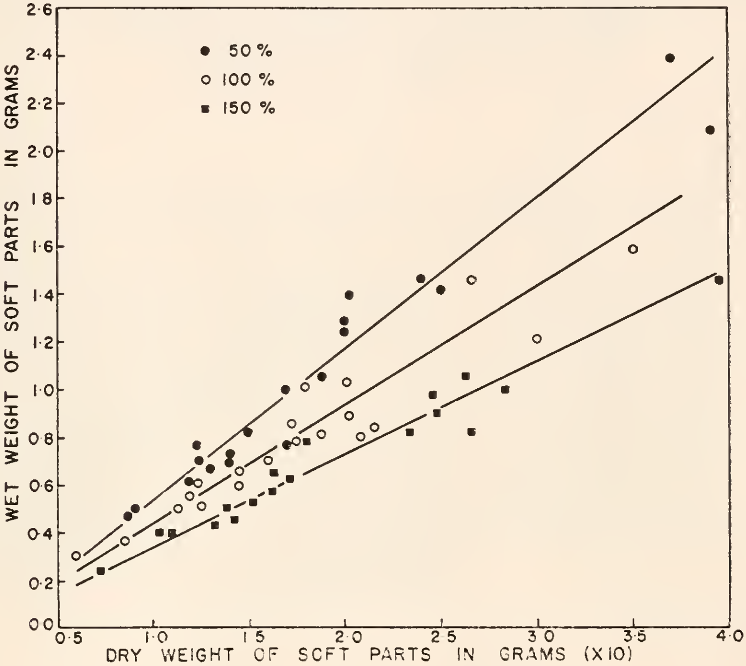

FIGURE 2. Relation between the wet-weight of soft parts and the dry-weight of soft parts, in grams, for *A. limatula* from Dike Rock, Scripps Institution. The points represent individuals maintained at 50, 100, and 150% sea water for 24 hours and *b* values are 0.63, 0.50, and 0.40, respectively.

Because of the absence of osmoregulation in animals from Dike Rock, Punta Banda animals were tested at two salinities (50 and 150% sea water) for no longer than a three-hour period. The osmotic responses of limpets from the two sides of Punta Banda and Dike Rock essentially are identical (Fig. 1).

Weight change experiments

Figure 2 is a plot of the wet-weight of soft parts as a function of dry-weight of soft parts after a 24-hour period at 50, 100 and 150% sea water. Analysis of these data has shown that the wet weight-dry weight relationship is best described by a straight line. Slope values (b), as determined by the method of least squares, for these lines are 0.63, 0.50, and 0.40 for 50%, 100%, and 150%, respectively. Comparison of any combination of two salinity regression lines has shown differences to be statistically significant ($P = 0.01$). Linearity of the curves shows that at each salinity there is a constant ratio of wet-weight to dry-weight over the weight range shown. Therefore, percentage body water shows no weight dependence at any of the salinities. The mean body water (expressed as percentage of body weight) has been determined, and is 78% for baseline animals from 100% sea water, 82% for animals from 50% sea water and 73% for animals from 150% sea water (Table I). Therefore, there was an average 28% *increase* in body water in 50% sea water and approximately the same *decrease* (28%) in 150% sea water when both are compared with the 100% sea water baseline. These calculations are based on the fact that for a given weight animal,

TABLE I

Changes in wet weight, dry weight and total water as per cent body weight in Acmaea limatula in 50%, 100% and 150% sea water. Total wet weight values were obtained before immersion in the experimental salinities, 50% and 150% sea water. Wet weight of soft parts (1) resulted when shell weight was subtracted from total wet weight. Wet weight of soft parts (2) were values obtained after 24 hours immersion in the experimental salinities.

50% SW					100% SW				150% SW				
Total wet weight (gms.)	(1) Wet weight soft parts (gms.)	(2) Wet weight soft parts (gms.)	Dry weight soft parts (gms.)	Total water % body weight	Total wet weight (gms.)	Wet weight soft parts (gms.)	Dry weight soft parts (gms.)	Total water % body weight	Total wet weight (gms.)	(1) Wet weight soft parts (gms.)	(2) Wet weight soft parts (gms.)	Dry weight soft parts (gms.)	Total water % body weight
1.4207	0.6472	0.7133	0.1352	81.04	3.0436	1.2236	0.3018	75.34	2.9028	1.3186	0.9898	0.2828	71.42
1.5450	0.7315	0.7739	0.1725	77.71	1.2995	0.6082	0.1453	76.10	4.4532	1.7805	1.4586	0.3952	79.90
1.3980	0.5790	0.6618	0.1283	80.61	1.9273	0.7782	0.1748	77.53	1.3180	0.6374	0.4705	0.1415	69.92
1.4146	0.6696	0.8147	0.1530	81.22	0.6928	0.3196	0.0613	80.81	1.0209	0.4618	0.3851	0.1070	72.21
1.3037	0.6037	0.6068	0.1171	80.70	1.5436	0.7073	0.1598	77.40	2.7523	1.2181	0.9756	0.2476	74.62
2.7468	1.1931	1.4266	0.2508	82.41	0.8514	0.3749	0.0846	77.43	2.2798	0.8765	0.7872	0.1779	77.40
2.3510	1.0437	1.2655	0.2017	84.06	2.0868	1.0176	0.1794	82.37	1.5970	0.7123	0.6284	0.1709	72.80
0.8396	0.4208	0.4790	0.0865	81.94	1.8631	0.8167	0.1877	77.01	2.6243	1.1586	0.8904	0.2476	72.19
2.3088	0.8665	1.0714	0.1707	84.06	1.9100	0.9144	0.2030	77.79	3.7538	1.5395	1.4618	0.3352	77.06
1.3292	0.6040	0.7077	0.1392	80.33	1.1983	0.5629	0.1208	78.53	2.1562	1.0776	0.8202	0.2338	71.49
0.9147	0.4074	0.4982	0.0895	81.96	1.6588	0.8425	0.2164	74.31	2.9687	1.2395	1.0636	0.2616	75.98
1.2538	0.5692	0.7081	0.1238	82.51	2.1856	1.0391	0.2018	80.57	2.1477	0.8917	0.8215	0.2657	67.65
2.0858	1.0010	1.2969	0.2034	84.31	1.0259	0.4963	0.1254	74.73	1.6088	0.7073	0.6566	0.1625	72.25
4.4188	1.7264	2.3998	0.3680	84.66	2.0205	0.8192	0.2079	74.62	1.0597	0.4649	0.3922	0.1025	73.86
1.0942	0.5563	0.7553	0.1230	83.71	3.5489	1.4679	0.2655	81.91	1.2498	0.5712	0.5108	0.1390	72.78
2.0348	0.8913	1.0694	0.1880	82.42	2.5193	0.8456	0.1728	79.56	1.6555	0.6627	0.5277	0.1511	71.36
2.4626	1.0270	1.4187	0.2071	85.40	1.2226	0.6255	0.1186	81.03	1.6388	0.6815	0.5650	0.1615	71.41
2.4067	1.2127	1.4654	0.2386	83.71	3.4640	1.5934	0.3513	77.95	0.6682	0.2968	0.2405	0.0731	69.60
					1.0167	0.4857	0.1144	76.44	1.1357	0.5198	0.4413	0.1307	70.38
					1.3939	0.6740	0.1454	78.42					
Mean —	—	—	—	82.38	—	—	—	77.99	—	—	—	—	72.86
S.D. —	—	—	—	1.92	—	—	—	2.49	—	—	—	—	3.02
σ_m —	—	—	—	0.45	—	—	—	0.56	—	—	—	—	0.69

	50%–100% S.W.	50%–150% S.W.	100%–150% S.W.
df	36	35	37
t	6.115	11.513	5.773
P	0.001	0.001	0.001

for example 100 grams, in 100% sea water, 78 grams are water and 22 grams are non-water. This latter value is essentially a constant. In 50% sea water, 82% of the animal is water and 18% is non-water, which in turn is 22 grams. Then, if 22 grams equals 18%, 82% equals 100 grams of water, or a gain of 22 grams, which is the 28% increase in the body water of the animal. The same argument must be considered for the animal in 150% sea water. In this instance there is a decrease in the total body water, but the non-water component remains constant. Therefore, the per cent increase and decrease in body water can be the same, although absolute mean body water can differ in the experimental salinities.

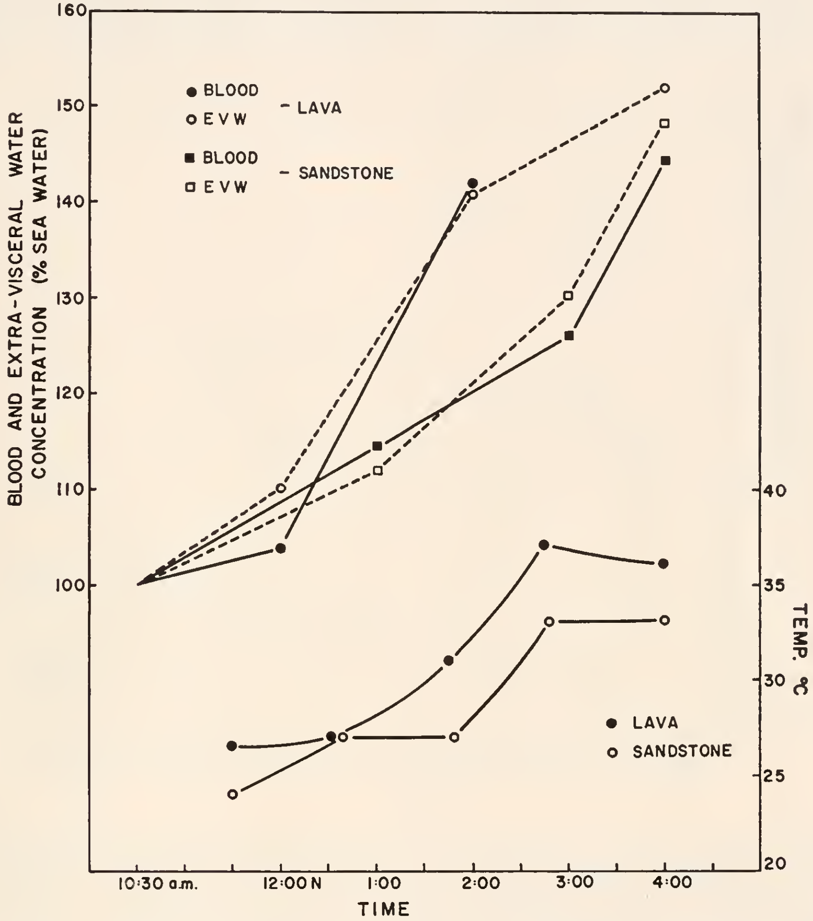

FIGURE 3. Change in concentration of blood and extra-visceral water of *A. limatula* placed on grey lava and grey sandstone out of water. Each point is the mean value for at least ten animals. Blood concentration at beginning of experiment was 99.4% sea water. Experiment was performed on July 13, 1960, at Dike Rock, Scripps Institution.

When the mean body water, expressed as per cent body weight of the three groups of animals in different salinities, is compared in all combinations, the differences are highly significant ($P = 0.001$, see Table I).

Weights of animals shown in Figure 2 and Table I are considered to be representative of the populations of this limpet, and the changes shown must be assumed to be representative of the response any individual would demonstrate if successive wet and/or dry weights of soft parts in two different salinities could be obtained.

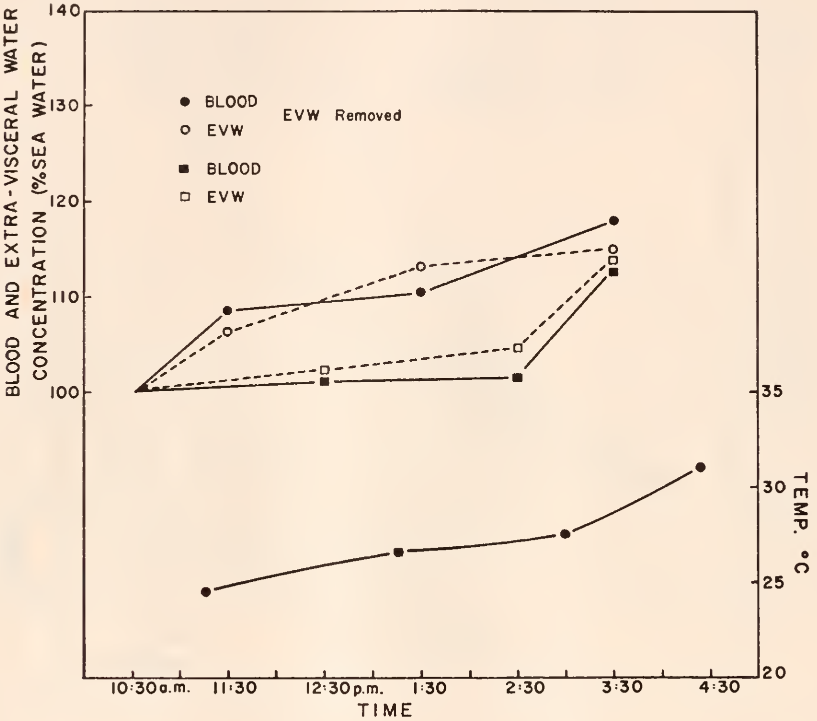

FIGURE 4. Change in concentration of blood and extra-visceral water of *A. limatula* when extra-visceral water was removed, as compared with *A. limatula* from which the extra-visceral water was not removed. Both groups were on grey sandstone out of water. Each point is the mean value for at least ten animals. Blood concentration at beginning of experiment was 99.6% sea water. Experiment was performed on July 15, 1960, at Dike Rock, Scripps Institution.

Total wet weight in Table I was measured before animals were immersed in the experimental salinities. Wet weights of soft parts (1) were body weights before immersion, and were determined by subtracting shell weight from total wet weight. Shell weight does not change and dry weight of shell differed from wet weight by less than 1%, and this difference was statistically insignificant. Shell weight (based on approximately sixty animals) represented 55.23% of total weight. Wet weight of soft parts (2) was determined after 24 hours immersion in the experimental salinities, 50% and 150% sea water.

Field desiccation experiments

Dike Rock

Figure 3 shows the effect of exposure of animals on two different substrates. It becomes apparent immediately that the rate of change of the concentration of the blood and extra-visceral water of animals on lava is much greater that that of animals on sandstone of the same color. The temperature record indicates that this more rapid rate of change on lava is a direct effect of the more rapid rise in temperature and higher values reached on the lava as compared with the sandstone. The rise in blood concentration parallels the rise in extra-visceral water for animals measured on sandstone. The same parallelism occurs with animals on lava. Based on the anatomy of the limpet, the extra-visceral water is in immediate contact with the environment and it would be expected, *a priori,* that a change in the extra-visceral water would occur first. In general this is what occurs in all three field experiments. At the last blood sampling from animals on the lava we were unable to obtain blood. However, extra-visceral water was still present. This suggests that with extreme desiccation there may be a movement of fluid from the sinuses within the animal to the extra-visceral space.

The remaining experiments were conducted on sandstone because this substrate is the more typical one both at Dike Rock and Punta Banda. Further, the rate of rise of temperature on the sandstone is slower than on lava. Figure 4 compares the response of a group of animals which have been shaken and damp-dried with that of a group of animals which have been placed directly on the substrate. The concentration of both extra-visceral water and blood was higher in the group which had a portion of the extra-visceral water removed. However, at the end of the experiment there was no difference in the concentrations of the extra-visceral water and blood of the two groups. The sudden increase in blood and extra-visceral water concentrations between 2:30 P.M. and 3:30 P.M. for the groups from which extra-visceral water was not removed was due probably to the rather abrupt temperature rise. Because of the differences in temperatures recorded for the two days at Dike Rock it is impossible to compare the results obtained from animals on sandstone for these two days.

Punta Banda

Comparison of the two populations of animals from opposite sides of Punta Banda under identical conditions of exposure showed that the blood and extra-visceral water concentrations of the Bay (Todos Santos) population increased more rapidly, remained at higher concentrations (a difference ($P = 0.01$) of 10% sea water in each case), than that of the Open Coast (Papalote) population (Fig. 5). This difference was evident throughout the period of exposure. It was noted that at the end of the experiment the two groups appeared very different. The Open Coast group had more fluid in the mantle tissues, and it was more difficult to obtain blood samples from the Bay group.

DISCUSSION

The data show that the intertidal gastropod *Acmaea limatula* does not osmoregulate over the range of salinities, from 25% to 150% sea water (Fig. 1). The relative blood concentration and weight changes shown for *A. limatula* in hypotonic media are similar to that reported for *Doris* (Bethe, 1934) and for *Onchiduim* (Dakin and Edmonds, 1931). However, *A. limatula* differs during the course of our experiments (48 hours) from that reported for *Aplysia* (Bethe, 1930). *Aplysia* shows an initial weight change in low and high salinities, but within a few hours approaches its original weight due to a subsequent gain or loss of ions. *Acmaea limatula* at 24 hours in 50% sea water shows a weight gain and in 150% sea water shows a weight loss (Fig. 2). In the same time period and salinities there is a corresponding increase and decrease in blood concentration which remains isotonic with the medium for an additional 24 hours. Although weight changes were not followed beyond 24 hours, it is probable that the body weight would not have returned to normal. However, Gross (1954) has shown that the sipunculid *Dendrostomum* remains isotonic in various sea water dilutions but can return to normal weight.

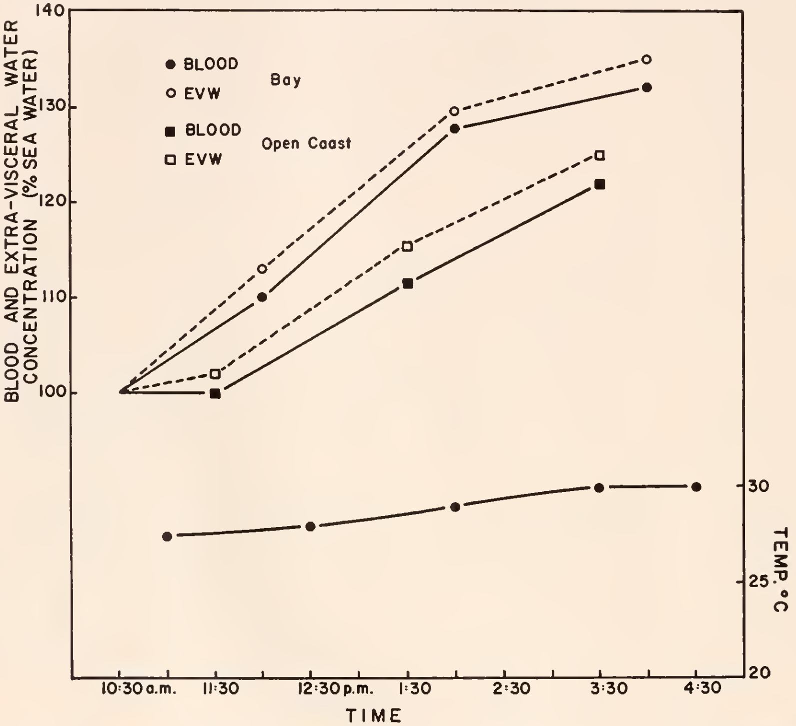

FIGURE 5. Change in concentration of blood and extra-visceral water of *A. limatula* from the Bay and Open Coast sides of Punta Banda, Baja California. Open Coast animals spent 24 hours in the Bay intertidal zone before experiment. Both groups were on light-colored sandstone. Each point is the mean value for at least ten animals. Blood concentration of both groups at beginning of experiment was 99.5% sea water. Experiment was performed July 28, 1960, at Todos Santos Bay, Punta Banda.

It has been demonstrated (van Weel, 1957) that *Aplysia juliana* can regulate weakly in 95% sea water. He criticized the results on *Aplysia* reported by Bethe

(1930) and has suggested that the low salinity (75%) to which the animals were subjected was a non-physiological condition. Further, van Weel contended that since weights of some animals, after osmotic equilibrium was established in 75% sea water, were less than initial weights, this condition reflected active water removal, and hence weak regulation. Van Weel also has re-evaluated results for *Doris* (Bethe, 1934) and interpreted failure to reach osmotic equilibrium in 75% sea water, as measured by blood chloride concentration, to mean that the blood remained hypertonic, and the animals showed some regulation.

Gross (1954) in his work on *Dendrostomum* has shown that this sipunculid responds superficially as an osmometer, and has discussed this isotonic relation of blood to various sea water concentrations, based presumably only on water fluxes, in a hypothetical manner, determining mobile (osmotically active) water by the following:

$$V = \frac{C_2 \quad (\Delta W)}{C_1 - C_2}$$

V is the volume of mobile water in per cent body weight (soft parts), C_1 is the concentration of body fluids in, for example, an external salinity of 100% sea water, and C_2 is body fluid concentration after exposure to a different external salinity, both expressed as per cent sea water, and ΔW is the change in weight after a steady-state is attained due to change in salinity. This relationship suggests whether blood concentration changes result from water or water and ion fluxes, when an external concentration change is effected.

Assume a volume of solution which is essentially constant among animals in 100% sea water, into which only water moves or leaves when the salinity of the external medium is changed. If the concentration change (C_1-C_2) of body fluids and the volume change (ΔW) of animals are known, one can calculate the original volume of the solution for the animal in 100% sea water. If only water were effecting the change, then the value for V would be the same whether the animal were in 50% or 150% sea water. However, if salts also were leaving or entering, values for animals in these two external salinities would differ.

In order to calculate mobile water values for each individual it is necessary to determine change in weight (ΔW) expressed as per cent body weight from one sea water concentration to another. Reference to the wet weights of soft parts (1) before immersion and (2) after immersion for either 50% or 150% sea water provides these data (Table I). C_2 may be obtained from Figure 1, blood concentration at 48 hours for 50% or 150% sea water. Determination of mobile water (V) follows from the above formula.

When this line of reasoning is applied to limpets in external salinities of 50% and 150% sea water, average mobile water values differ. For 50% sea water, mobile water, expressed as per cent original body weight (soft parts), equals 27.8%; for 150% sea water, 43.7%. The differences in mobile water values at these two salinities are statistically significant ($P = 0.01$). This would be interpreted to mean that salts play a greater role (at least percentage) in effecting an osmotic pressure change in the blood in 50% sea water than in 150%. In 50% sea water, salts leave and water enters the blood. If no salts were exchanged, mobile water values for both salinities should be the same, and the limpets would be responding to external salinity changes as an osmometer. In the absence of other data, however, one cannot state definitely for animals in 150% sea water that salts are entering, as well as water leaving. Comparison of osmotically active water for a sipunculid (Gross, 1954) in high salinity water with that for the limpet in similar water shows the mobile water value for the limpet to be considerably lower. This would suggest that salts are entering the limpet in 150% sea water, thus reducing ΔW for a given concentration change. Data for the sipunculid show mobile water values for high and low salinity media to be the same.

Percentage change of body weight as water in *A. limatula* is approximately the same, 28%, in hypo- and hypertonic media. These values generally are of the same magnitude as those reported in the literature. Bethe (1930) showed that the weight of *Aplysia* increased approximately 50% in two to four hours in 50% sea water, and 20% in 75% sea water over the same time period. Dakin and Edmonds (1931) showed for *Onchidium* an 8% increase in weight in one and one-half hours at 50% sea water, and a 70% increase in 22 hours in 10% sea water.

Under conditions of air desiccation the response is very similar to that recorded for salinity dehydration. This can be seen by comparing Figure 1, three-hour, 150% sea water with Figure 3, 4:00 P.M. sample. Certainly, air desiccation is the more natural environmental stress, although in high tide pools it is likely that high salinities can be reached. Individuals of *A. limatula* living relatively high in the intertidal zone can easily withstand periods of desiccation due to tidal exposure. Body fluid concentration rises appreciably, but data from Figure 1 show that animals in 150% sea water survived for two days. We were able to collect extra-visceral water, but not blood, from limpets on lava at 4:00 P.M. The animals were shrunken, and no response to stimuli was evoked. The animals appeared to be moribund. We had no difficulty collecting blood and extra-visceral water from limpets on sandstone. The temperature on the sandstone was 5° C. lower. It seems reasonable to suggest that the higher temperature on the lava was the cause of death. Certainly, it would appear to be advantageous for *A. limatula* to frequent the sandstone as opposed to the lava, and so they do. The only conditions under which *A. limatula* were found on lava were when the lava was protected from the sun by an overhang or the limpets were in pockets of water.

The question remains whether extra-visceral water serves any osmotic function. It does appear to serve an osmotic function in that, if the animal does not have this water jacket, the body would lose water to the space more rapidly and the blood concentration would, therefore, rise more rapidly (Fig. 4). The animals in which the extra-visceral water was removed demonstrate this. At the same time, the water jacket has another function, which may well be the primary one, to serve as a temperature buffer. Since the increase in extra-visceral water and blood concentration is a direct function of the temperature (through evaporation), the differences in fluid concentration between the two groups must be due to the presence or absence of extra-visceral water.

We noted, during the later stages of desiccation, on July 13, that the limpets raised their shells off the substrate. This behavioral response was more evident among the animals on the lava (the warmer substrate). This behavior may be interpreted in two ways: an escape response or a cooling response. We are inclined to believe that it is not an escape response because we observed no animal movement approximately one-half hour after placing the animals on the rocks.

This response resembles the "stilting response" described by Alexander and Ewer (1958) in the scorpion. They concluded that the scorpion used this behavior for cooling. It may well be that this is the situation in *A. limatula*. When the animal raises its shell, the extra-visceral water surface is presented to the air. Evaporation, and, therefore, cooling, may now take place.

It is clear that the population from Todos Santos Bay and the population from the Open Coast (Papalote) did not respond similarly to identical desiccating conditions. The blood and extra-visceral water of individuals from the Bay were more concentrated throughout the experimental period. The reason for this difference is not clear. It is possible that the difference in the two populations from Punta Banda is due to a behavioral response. The Bay population is exposed generally to higher temperatures. These limpets may rise from the substrate more often for evaporation, thus losing water more rapidly. However, this behavioral response described for animals from Dike Rock was not observed at Punta Banda.

The Bay and Open Coast habitats, where these populations were collected, are different. The Bay is a warm-water body (during the time of our work the temperature ranged from 22.5° to 25° C.), with considerably reduced wave action, no macroscopic flora, reduced numbers of common intertidal species, and few individuals of those species that were present (*A. limatula*, in contrast, was abundant). All substrate was light-colored sandstone, and the salinity, during the days of our work, ranged from 85 to 90% sea water. The Open Coast habitat, on the other hand, is an area of cold water upwelling (14° C.), vigorous wave action, abundant macroscopic flora, numerous intertidal species with the individuals in great numbers, a mixed substrate of darker sandstone and lava, and salinity range from 95 to 100% sea water. The temperature differences we have recorded, although not as great during the winter months, are found throughout the year (see Material and Methods).

Pickens (personal communication) has shown an acclimation of the heart rate to temperature in populations of *Mytilus californianus* from the two sides of Punta Banda. Differences in response to short-term desiccation of the two populations of *A. limatula* may also be a reflection of the differences in temperature on the two sides. Unfortunately, there are no long-term salinity data available. We are not implying that the small differences in salinity, that we have recorded, are significant in terms of the response pattern. But we cannot discount the possible role of a difference in salinity in terms of its interaction with temperature. This has been brought out by the work of Kinne (1956) on growth and reproduction in the hydroid *Cordylophora*, Todd and Dehnel (1960) on temperature tolerance, and Dehnel (1960) on respiration in the intertidal crabs *Hemigrapsus nudus* and *H. oregonensis*. If the osmotic differences, resulting from desiccation, between the two Punta Banda populations are phenotypic, it is possible that such differences would be greater if the Open Coast group had not spent approximately 24 hours in the Bay intertidal zone. The response of the Open Coast limpets, due either to an acclimation or an environmental stress, following transfer to the Bay intertidal zone would seem only to shift in the direction of that response shown by the Bay animals, *i.e.*, the curve for the Open Coast population would approach that of the Bay group.

SUMMARY

1. Osmotic and air desiccation experiments were conducted on three populations of *Acmaea limatula*: Dike Rock, Scripps Institution of Oceanography; Todos Santos Bay, Punta Banda, Baja California; Papalote, Punta Banda, Baja California.

2. *A. limatula* does not osmoregulate over a range of salinities from 25 to 150% sea water. Mobile water calculations show that blood concentration changes in different external salinities are effected by salt as well as by water movement, particularly at lower external salinities.

3. Body water as percentage body weight decreased 28% in 150% sea water and increased 28% in 50% sea water in 24 hours.

4. Isotonicity of the blood with the medium is reached within three hours in hypertonic media; longer periods were necessary in hypotonic media.

5. Under identical conditions of exposure, the rate of rise of blood and extra-visceral water concentration of animals on lava was faster and reached a higher concentration when compared with animals on sandstone.

6. The effect of removal of the extra-visceral water resulted in an increased concentration of both the extra-visceral water and the blood, when compared with limpets in which the extra-visceral water was not removed.

7. The two Punta Banda populations, under identical conditions of exposure, were different; the Bay population showed a more rapid increase in extra-visceral water and blood concentration than did the Open Coast population.

8. Under conditions of exposure, the extra-visceral water appears to function both in an osmotic and temperature buffering capacity.

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RESPONSES OF SPECIFIC NEUROSECRETORY CELLS OF THE COCKROACH, BLABERUS GIGANTEUS, TO DEHYDRATION[1]

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So much attention has been given to the regulation of water content in vertebrates (Sawyer, 1961) it is surprising that so little work has been done concerning the same processes in insects. The limited information that has accumulated about osmoregulation in insects is in part conflicting (see Discussion). Furthermore, as is generally true in all kinds of endocrine studies on insects, workers have confined their attention to the brain and corpora cardiaca-allata complex as possible sources of endocrines of water metabolism (Day, 1943; Stutinsky, 1953; Altmann, 1956; Raabe, 1959), in spite of the fact that the vast majority of neurosecretory cells in some insects are in the ventral ganglia (Geldiay, 1959; Füller, 1960).

This investigation was undertaken to observe by histological methods the entire complement of neurosecretory elements in the major ganglia of the cockroach, *Blaberus giganteus*, under normal and dehydrated conditions, to see if changes in certain ones could be detected, and thus perhaps implicate them in osmoregulation. Specific responses were observed in the neurosecretory cells of the pars intercerebralis and corpora cardiaca, which confirms the observations of others (Pflugfelder, 1937; Nayar, 1957), and in the type A cells in the three thoracic ganglia, an observation that has not been reported previously.

MATERIALS AND METHODS

The experiments utilized adult, male *Blaberus giganteus* L.[2] Prior to use the animals were maintained in mixed colonies of about 25 animals each in an animal room which was illuminated from 6 A.M. to 8 P.M. E.S.T. They were provided at all times with Purina lab chow and water. Male roaches were picked randomly from the colonies and placed individually in quart Mason jars with lids in which screen-wire discs were substituted in the retaining rings for the inserts. Four groups were set up:

Group	Food	Water
A	+	+
B	−	+
C	−	—
D	+	−

Groups A and B received a vial of water plugged with cotton. Groups A and D were given a pellet of lab chow.

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[2] Cockroaches for establishing colonies were obtained from the Gulf Research and Development Company, Harmarville, Pennsylvania.

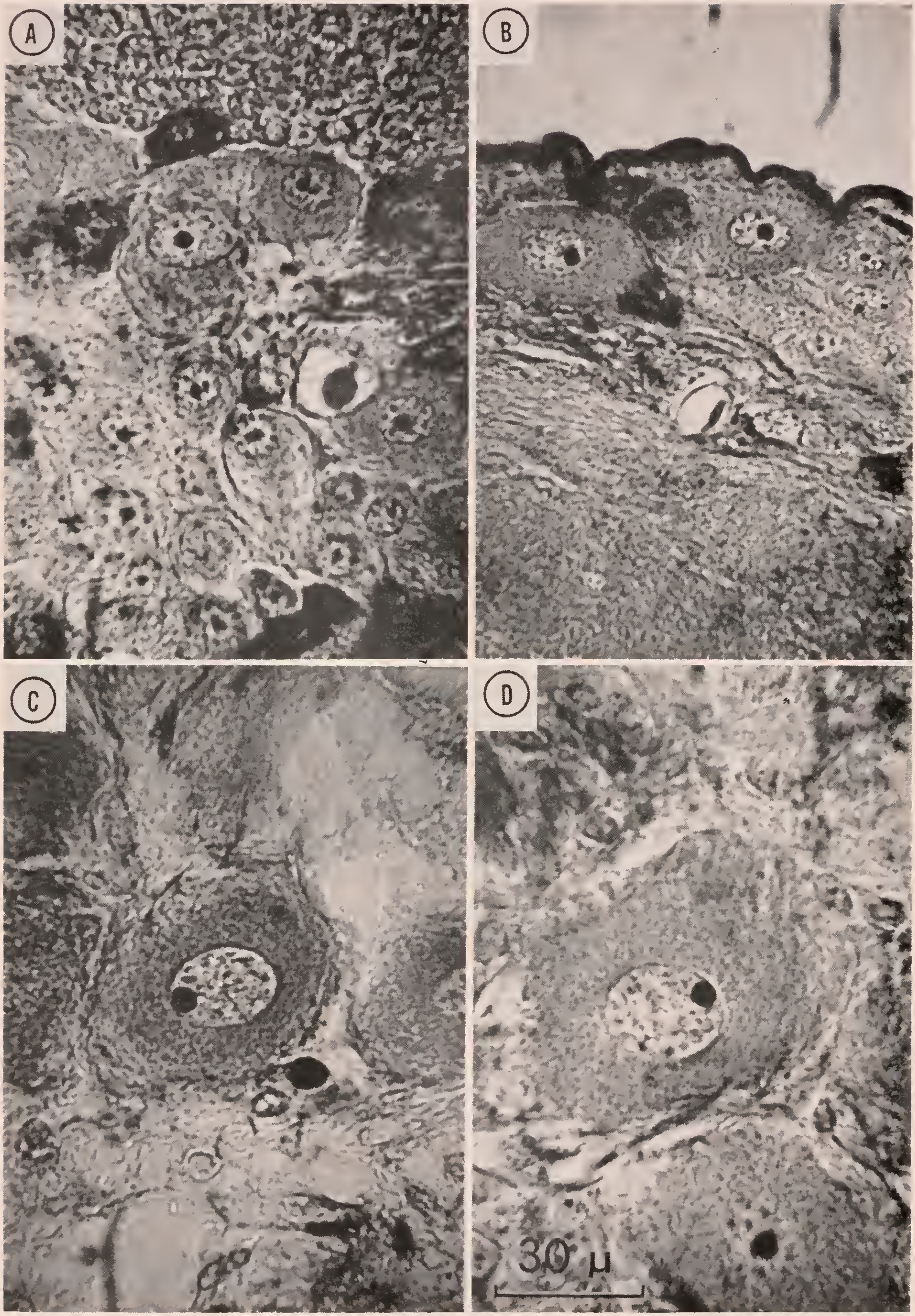

FIGURE 1.

An animal from each group was sacrificed at 4 P.M. on different days after the start of the experiment up to 12 days. All animals were sacrificed by an injection of Bouin's fixative (modified by Halmi, 1952) into the hemocoel and the nerve cord was carefully dissected out in saline solution (Hoyle, 1953). The tissue was fixed in Bouin's, washed, embedded, sectioned at 10 microns and then stained with either the aldehyde fuchsin (AF) of Gomori-Halmi-Dawson (Dawson, 1953) or Mallory's triple stain (Gray, 1954).

To compare the effects of NaCl injection with those of dehydration, other animals were injected with 4 × NaCl in Hoyle's solution at 4 P.M. and then sacrificed after 24 hours. The nervous tissue was treated in the same manner as just described.

RESULTS

A total of 75 animals were used in seven replications of the basic experiment. In three experiments the cockroaches were maintained for 3, 5, 7, and 9 days under the experimental conditions. In one experiment animals were sacrificed at 5, 8, and 12 days, while in three other experiments they were sacrificed at 5 and 6, 5 and 7, and 5 and 8 days only. The results of all the experiments are in good agreement. (Although Mallory's triple stain was used in one experiment, all the observations are based on AF staining.)

The animals deprived of water showed loss of weight, 0.05–0.1 g. per day (about 1–2% of total body weight), while those given water did not change weight significantly. No differences could be detected between Groups A (with food) and B (without food), both of which were given water. Also, the effects of dehydration were the same for C and D (both without water), although Group C was not given food. Thus, it appears that nutritional states cannot account for the results observed in Groups C and D.

The effects of dehydration were quite pronounced after five days in both Groups C and D, producing specific effects on type A neurosecretory cells (Füller, 1960) of the pars intercerebralis and thoracic ganglia. Specific changes were not detected in any of the type A neurosecretory cells of the subesophageal or abdominal ganglia, or in any other neurosecretory cell types (Fig. 1). Periods of dehydration longer than 5 days, however, tended to produce non-specific alterations in the entire neural structure.

The smaller, or α-type, A cells of Füller (1960) (type I cells of Geldiay, 1959) in the pars intercerebralis of the supraesophageal ganglion showed specific cytological changes under dehydration. These cells in a dehydrated animal appear intensely and almost homogeneously purple after AF staining as compared to the purplish, more granular cytoplasm of the normal animal (Fig. 2). Also, the axons of the cells of the pars intercerebralis show a much greater amount of stainable substance in the fiber tracts, when compared to the appearance of comparable sections from control animals. In the corpora cardiaca of dehydrated animals there is a comparable accumulation of stainable material (Fig. 3).

FIGURE 1. Type B neurosecretory cells from those of normal and dehydrated animals. A, pars intercerebralis of normal animal (Group A); B, pars intercerebralis of dehydrated animal (Group D); C, thoracic ganglion of normal animal (Group A); D, thoracic ganglion of dehydrated animal (Group D). Cells from dehydrated animals do not differ from normal animals.

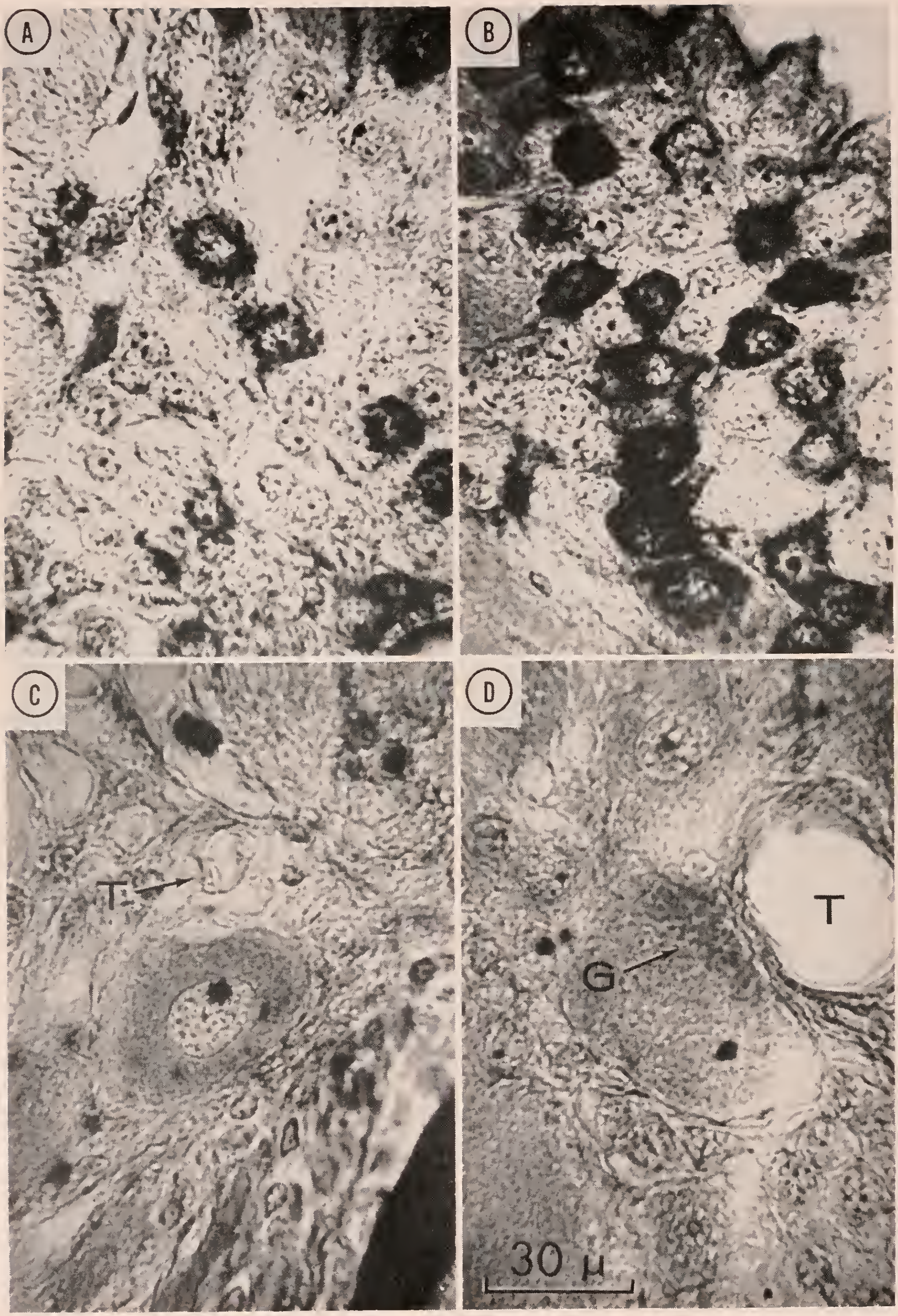
A
B
C
T
D
G
T
30 μ

FIGURE 2.

The kind of A cell found in the thoracic ganglia, the larger, so-called β-type (Füller, 1960), is affected in quite a different manner. (There are about four of these cells in each ganglion.) In dehydrated animals their cytoplasm appears to be coagulated and relatively depleted of stainable granules, except for a dense mass of material clumped to one side of the cell. These cells always appear to be closely associated with a prominent trachea and the site of clumping is always toward the cell boundary adjacent to the trachea (Fig. 2).

No other cytological changes, such as nuclear or nucleolar size or morphological differences, could be detected in the A cells of either the pars intercerebralis or thoracic ganglia.

Discussion

It is assumed that the stainable granules are neurohormones or, more likely, the protein carriers of the hormones (Sloper, 1958). Accumulation of granular material by the type A cells of the pars intercerebralis may be variously interpreted. It could mean that the cells are sources of an antidiuretic factor, as evidenced by their great activity—granule production—when desiccated. On the other hand, a diuretic factor may be secreted which, to allow water conservation, is retained—hence, granule accumulation. The latter interpretation agrees with that of Nayar (1957) who forcibly fed *Iphita limbata* (Hemiptera) salt water and found that the pars intercerebralis neurosecretory cells were "loaded with stainable colloids" and "release of neurosecretory material to the blood appears to be inhibited." Also, animals under conditions that cause them to take up water are described as having "comparatively colloid-free neurosecretory cells."

However, Nayar in a later work (1960) gives another description of the neurosecretory cells of the same animal. Those subjected to salt-water feeding or injections are said to show a "characteristic clumped pattern" of the granules and the nucleoli are described as "highly distorted" and "very much shrunk." Furthermore, insects which took up water because of increased cuticular permeability are described, in contrast to the 1957 report, as having cells which "showed a very dense mass of stainable colloids in their cytoplasm." The secretory matter is in this paper interpreted as anti-diuretic in nature. These two reports appear to be conflicting.

The cytological observations in the present study agree with those of Pflugfelder (1937) who reported that the corpora cardiaca of desiccated phasmids showed a large accumulation of granules.

Certain evidence from several investigators seems to favor the view that the brain and retrocerebral complex produce an anti-diuretic hormone. Stutinsky (1953) delayed urine elimination in the rat with injection of extracts of the pars intercerebralis or of the corpora cardiaca and allata of *Blabera fusca*. Altmann's experiments (1956) with the honeybee and Raabe's work (1959) on phasmids support the existence of an anti-diuretic factor in the corpora cardiaca.

FIGURE 2. Type A neurosecretory cells from those of normal and dehydrated animals. A, pars intercerebralis of normal animal (Group A); B, pars intercerebralis of dehydrated animal (Group D); C, thoracic ganglion of normal animal (Group A); D, thoracic ganglion of dehydrated animal (Group D). Dehydrated animals show a great accumulation of granules in the cells of the pars intercerebralis and in the thoracic ganglia the cells have clumps of granules (G) adjacent to the trachea (T).

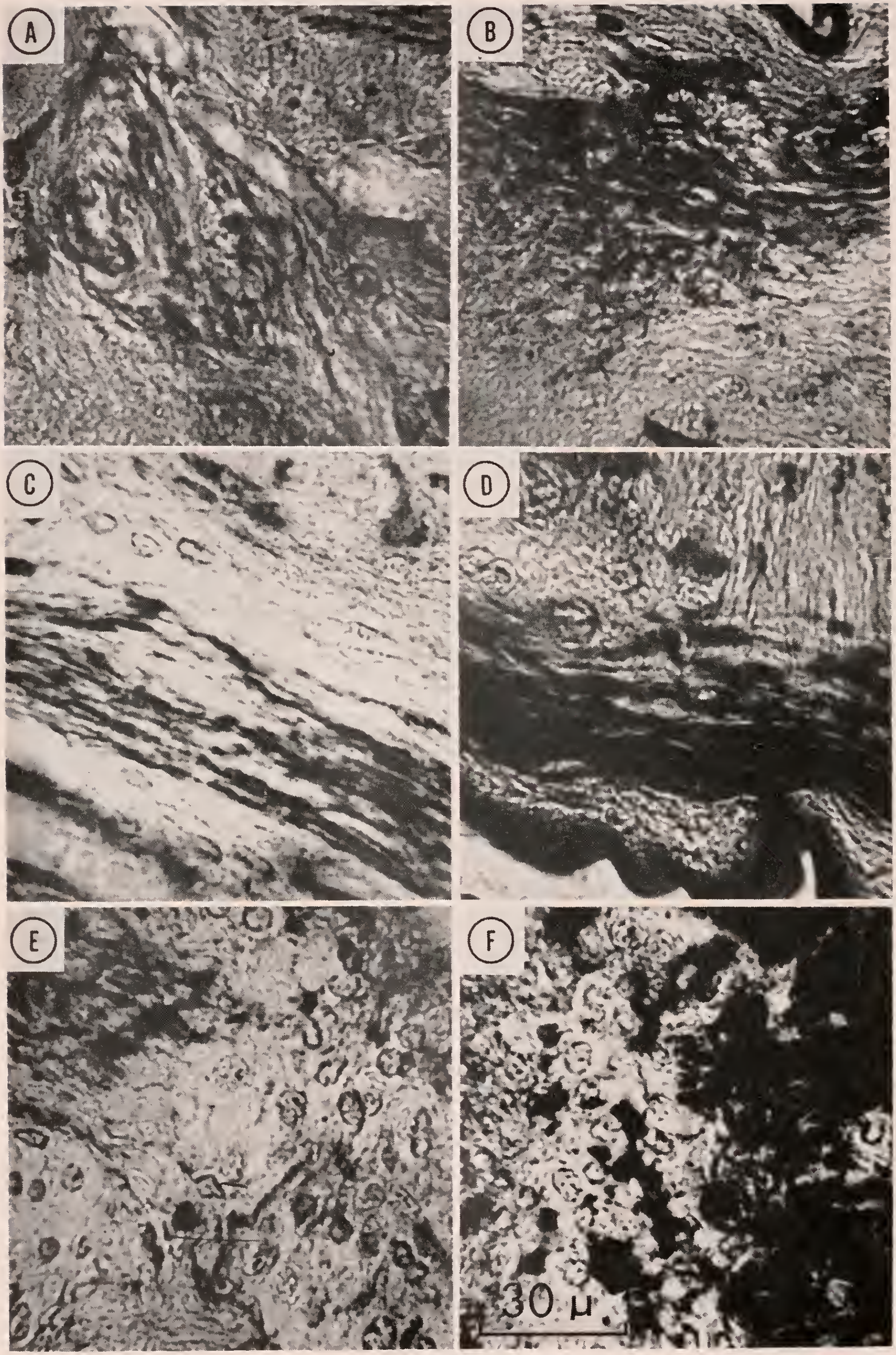

FIGURE 3.

However, other evidence indicates a diuretic principle in the retrocerebral complex. Nuñez (1956) interprets his results with a coleopteran (*Anisotarsus*) as indicating a diuretic principle in the dorsal part of the brain and corpus cardiacum, based on the fact that if these parts are excised the abdomen swells with increased fluid. Day (1943) has observed polydipsia and bloating in a few flies after removal of the corpus allatum. Dethier and Evans (1961) have pointed out, however, that allatectomy could have caused injury to the recurrent nerve. They show that cutting this nerve causes polydipsia and bloating in flies, but were not able to produce these effects as a result of allatectomy. Removal of the medial neurosecretory cells in a few cases caused bloating, but this is not interpreted as evidence for a hormonal mechanism since these cells are also in neural contact with the recurrent nerve. However, Altmann (1956) found that extracts of the corpora allata increase water uptake by honeybees and also increase the excretory rate of Malpighian tubules.

Emerging from this confused assortment of information is the general impression that the brain and retrocerebral complex probably produce both anti-diuretic and diuretic factors, which may or may not be confined to specific cell groups or endocrine structures. In all probability there are species differences that contribute to some of the confusion.

As for the observations regarding changes in the A cells of the thoracic ganglia, little can be said in the way of interpretation except that they may be involved with osmoregulation. There are no reports in the literature known to the authors which relate osmotic regulation to the thoracic ganglia. No explanation for the peculiar clumping pattern can presently be offered, but since the site of clumping is always associated with a trachea some relationship to gas-diffusion gradients may be indicated. The fact that cytological responses to states of hydration are observed in the brain, and water-regulating hormones must surely be there, argues for the probability that the thoracic ganglia similarly will be found to be sources of such hormones. Current studies on other hormonal systems of the cockroach (Ralph, 1962), showing the widespread segmental distribution of hormones, provide strong evidence, by analogy, that certainly some of the ventral cord ganglia are involved with water-regulation hormones.

The interesting results of Nuñez (1956) on abdominal cord sectioning could actually be interpreted to mean that abdominal ganglia are sources of diuretic hormone, which fails to be released when severed from the central nervous system. (We found no cytological evidence for such a role, however.) Nuñez interprets the abdominal ganglia instead as sites of sensing devices which respond to swelling of the abdomen upon intake of fluid and trigger release of diuretic hormone from the brain. The concepts of Dethier and Evans (1961), most interestingly, appear to place these mechanoreceptors in the head vicinity and, furthermore, they suggest a strictly neural regulation of water intake.

FIGURE 3. Corpora cardiaca and fiber tracts between the pars intercerebralis and corpus cardiacum of normal and dehydrated animals. A, transverse section of tracts in normal animal (Group A); B, transverse section of tracts in dehydrated animal (Group D); C, longitudinal section of tracts in normal animal (Group A); D, longitudinal section of tracts in dehydrated animal (Group D); E, corpus cardiacum of normal animal (Group A); F, corpus cardiacum of dehydrated animal (Group D). Greater amounts of granular material are seen in the dehydrated animals.

The literature, obviously then, provides a confusing background for attempting to interpret the cytological findings of this investigation. Appropriate assays of all neurosecretory structures will be required before it can be concluded how many water-regulating hormones there are, in what neural structures they are found, and what their actions may be. Work in this laboratory is currently proceeding along lines that may help clarify some of these aspects.

SUMMARY

Regulation of water content in the cockroach, *Blaberus giganteus,* may be influenced by the secretions of specific neurosecretory cells. Under conditions causing dehydration (a) the type A cells of the pars intercerebralis show greater than normal amounts of stainable granules, and (b) the type A cells of the thoracic ganglia show clumping of granules toward the surface adjacent to a trachea and have a coagulated-appearing cytoplasm. Salt injections of 4 × NaCl in Hoyle's solution produced the same cytological appearances as dehydration. Specific cytological changes were not seen in any other neurosecretory cells.

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